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*Estrogens as Endogenous Carcinogens
in the Breast and Prostate*

2000
Number 27

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Elizabeth A. Hart	

Dedication

This monograph is dedicated to the memory of Ms. Carol Hochberg, a member of the symposium discussion entitled “Panel Perspective from the Advocacy Community,” and all of the family members and friends of the participants whose lives have been lost to cancer.

Preface

Ercole Cavalieri, Eleanor G. Rogan

In Greek mythology, the great Athenian hero Theseus volunteered to go into the Labyrinth to slay the Minotaur, the monster that was satisfied only by devouring Athenian youths. This noble accomplishment could never have been achieved unless Ariadne had fallen in love with the handsome Theseus and followed him into the Labyrinth, leaving behind her a thread that marked their path through the tortuous passageways and blind alleys. The thread served not only to ultimately reach the Minotaur but also to find the way out of the Labyrinth once the Minotaur was slain. This mythologic story contains elements that illustrate the war against cancer and the subtle approaches needed to conquer it.

We can identify the Labyrinth as the series of complex and intertwined events leading to cancer, the Minotaur. The thread represents the fundamental physicochemical properties of the cancer-initiating molecules that allow us to unravel the primary events in this disease process, thus exiting the Labyrinth victorious.

The first landmark finding was obtained by the brilliant intuitions of James and Elizabeth Miller in the late 1960s who recognized first, that chemical carcinogens bind covalently to cellular macromolecules, and second, that the ultimate electrophilic species of chemical carcinogens react with nucleophilic groups of DNA, RNA, and protein. The importance of this finding was the identification of the reactive electrophilic species as the unifying factor among the different structures of chemical carcinogens. Subsequently, attention was focused on the carcinogen-induced alterations of DNA because of the critical, heritable function of this macromolecule. Reaction of chemical carcinogens with DNA led to the discovery of two types of adducts, the stable adducts that remain in DNA unless removed by repair and the depurinating adducts that are lost from DNA by destabilization of the glycosyl bond between deoxyribose and adenine or guanine. Experimental evidence indicates that depurinating adducts play the major role in generating the critical mutations leading to initiation of cancer. In turn, the depurinating adducts

obtained from electrophilic metabolites of estrogens, namely, catechol estrogen-3,4-quinones, may be endogenous initiators of several types of human cancer, including breast and prostate cancers.

Interest in the role of endogenous estrogens as tumor initiators led to the organization of a symposium on "Estrogens as Endogenous Carcinogens in the Breast and Prostate." The symposium, held March 16 and 17, 1998, explored our understanding of estrogen metabolism and genotoxicity and current knowledge in estrogen receptor-mediated processes as critical events in the initiation and promotion of cancer. Rather than merely reporting the proceedings of the symposium, this monograph represents an overview of various aspects of estrogen carcinogenesis, organized into comprehensive chapters by the symposium presenters.

As editors of this monograph, we wish to thank the Division of Cancer Biology, National Cancer Institute, in particular, Dr. David G. Longfellow and Ms. Karen R. Grotzinger, for the outstanding support given to the symposium and monograph. We also thank the members of the National Cancer Institute-supported focus group on estrogen carcinogenesis known as The Cancer Cube for their hard work and cooperation in preparing this monograph. Finally, we gratefully acknowledge the contributions of Mr. Harry W. Bullerdiek and Ms. Aimee R. Welch-Miller for coordinating all parts of the monograph preparation.

Omaha, Nebraska
August 17, 1999

Affiliation of authors: Eppley Institute, University of Nebraska Medical Center, Omaha.

Correspondence to: Ercole Cavalieri, D.Sc., Eppley Institute for Research in Cancer and Allied Diseases, 986805 Nebraska Medical Center, Omaha, NE 68198-6805 (e-mail: ecavalie@unmc.edu).

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Agenda

Estrogens as Endogenous Carcinogens in the Breast and Prostate

Westfields International Conference Center
14750 Conference Center Drive
Chantilly, Virginia 22021
March 15–17, 1998

Monday, March 16, 1998—Morning

8:00 a.m. Dr. David Longfellow Welcoming Remarks

Overview of Estrogens as Endogenous Carcinogens

8:10 a.m. **Introduction and Remarks** Co-chairs: **Dr. David Longfellow**
Dr. Richard Santen

8:25 a.m. Cellular and Molecular Interactions in Breast Cancer: Role of Estrogen and Its Receptors Dr. Jose Russo

9:25 a.m. Endogenous Estrogens as Carcinogens Through Metabolic Activation Dr. James Yager

10:25 a.m. **Break**

Estrogens as Endogenous Genotoxic Agents: DNA Adducts and Mutations

10:45 a.m. **Remarks** Co-chairs: **Dr. Joachim Liehr**
Dr. Ercole Cavalieri

10:55 a.m. Oncogenic Mutations by Depurinating Carcinogen-DNA Adducts Dr. Eleanor Rogan

11:35 a.m. Catechol Estrogen-3,4-Quinones and Apurinic Sites in Cancer Initiation Dr. Ercole Cavalieri

12:15 p.m. Lunch

Monday, March 16, 1998—Afternoon

Estrogens as Endogenous Genotoxic Agents: DNA Adducts and Mutations (continued)

1:15 p.m. Endogenous Oxidants and DNA Damage Dr. Krystyna Frenkel

1:55 p.m. Estrogen-induced Gene Mutations Dr. Deodutta Roy

2:25 p.m. Discussion and Summary

Tissue-Specific Synthesis and Oxidative Metabolism of Estrogens

2:40 p.m. **Remarks** Co-chairs: **Dr. James Yager**
Dr. Colin Jefcoate

2:45 p.m. Estrogen Formation by Aromatase in Breast Tissue Dr. Richard Santen

3:35 p.m. Break

4:05 p.m. Metabolic Activation of Estrogens by 4-Hydroxylation Dr. Joachim Liehr

4:45 p.m. Estrogen 4-Hydroxylation by Cytochrome P4501B1 Dr. Thomas Sutter

5:15 p.m. Discussion and Summary

5:30 p.m. **Adjournment Day 1**

Tuesday, March 17, 1998—Morning

Estrogen Metabolism by Conjugation

8:00 a.m.	Remarks	Co-chairs:	Dr. Richard Weinshilboum Dr. Julius Axelrod
8:15 a.m.	Methylation of Catechol Estrogens by Catechol- <i>O</i> -methyltransferase (COMT)		Dr. Cyrus Creveling
8:30 a.m.	COMT Genetic Polymorphism and Breast Cancer		Dr. Patricia Thompson
8:45 a.m.	COMT, CYP17, SRD5A Polymorphisms in Breast and Prostate Cancer		Dr. Douglas Bell
9:15 a.m.	Discussion and Summary		

Estrogen Receptor-Mediated Processes in Normal and Cancer Cells

9:30 a.m.	Remarks	Co-chairs:	Dr. George Stancel Dr. Robert Dickson
9:45 a.m.	Dissection of the ER Signaling Pathway: Insights into the Mechanism of Tamoxifen Resistance		Dr. Donald McDonnell
10:25 a.m.	Break		
10:45 a.m.	Investigating the Role of ER- α in Carcinogenesis Through the Use of Transgenic Mouse Models with Altered Levels of Receptor Expression		Dr. John Couse
11:25 a.m.	Regulation of the Cell Cycle and Cell Death in Mammary Cancer		Dr. Robert Dickson
12:05 p.m.	Lunch		

Tuesday, March 17, 1998—Afternoon

Estrogen Receptor-Mediated Processes in Normal and Cancer Cells (continued)

1:05 p.m.	Estrogen Receptor Structure, Modulators, and Targets in Hormone Responsive Tissues and Cancer		Dr. Geoffrey Greene
1:45 p.m.	Structure and Function of Estrogen Receptor- β		Dr. Jan-Ake Gustafsson
2:15 p.m.	Discussion and Summary		
2:30 p.m.	Break		

Estrogens and Cancer in Human Populations

2:50 p.m.	Remarks	Co-chairs:	Dr. Louise Brinton Dr. Shuk-mei Ho
2:55 p.m.	Estrogen Levels and Breast Cancer Risk		Dr. Paolo Toniolo
3:35 p.m.	Study Design Considerations in the Assessment of Cancer Risk in Relation to Genetic Polymorphisms		Dr. Montserrat Garcia-Closas
3:55 p.m.	Estrogens and Estrogen Metabolites: Technical Hurdles in Population Studies		Dr. Susan Hankinson
4:15 p.m.	DNA Biomarkers for Predicting Human Breast, Ovarian and Prostate Cancer		Dr. Donald Malins
4:45 p.m.	Discussion and Summary		

Panel Perspective from the Advocacy Community

5:00 p.m.	Introductory Remarks	Chair:	Ms. Elizabeth Hart
5:10 p.m.	Questions and Answers	Moderator:	Dr. David Longfellow
	Panelists:		
	Ms. Elizabeth Hart		
	Dr. Edison Liu		
	Dr. Richard Santen		
	Ms. M. Brooke Moran,		
	American Foundation for Urologic Disease, Inc.		
	Mr. Winston Dyer,		
	Association for the Cure of Cancer of the Prostate		
	Ms. Carol Hochberg,		
	Self-Help for Women With Breast or Ovarian Cancer		
	Ms. Diana Rowden, Komen Foundation		
6:00 p.m.	Meeting Adjournment		

Christine B. Ambrosone

Dr. Ambrosone is a Research Epidemiologist in the Division of Molecular Epidemiology at the National Center for Toxicological Research, Jefferson, AR. After receiving her undergraduate degree in medical anthropology from State University of New York (SUNY) Buffalo, NY, in 1989, she attended Roswell Park Cancer Institute (Buffalo, NY), where she completed her Master's degree in Interdisciplinary Natural Science. She received her Ph.D. in Cancer Epidemiology in 1994 from Roswell Park Cancer Institute, SUNY at Buffalo.

Dr. Ambrosone serves as an Assistant Professor in the Department of Pharmacology and Toxicology at the University of Arkansas for Medical Sciences; as Associate Editor, *Cancer Epidemiology Biomarkers and Prevention*; as Vice-Chairperson, Molecular Epidemiology Group of the American Association for Cancer Research; and as an Education Committee Member for the American College of Epidemiology. She has authored 23 papers, three conference proceedings, and five book chapters. Her honors include graduating Phi Beta Kappa, summa cum laude from SUNY in 1989, receiving a National Cancer Institute predoctoral fellowship at the Roswell Park Cancer Institute (1989), a Women in Cancer Research Brigid G. Leventhal Training Award (1995), an American College of Epidemiology National Student Prize Paper Award (1995), and a Food and Drug Administration Outstanding Achievement Award (1997).

Trained as a cancer epidemiologist, Dr. Ambrosone's primary research interests are in the role of interindividual genetic variability in endogenous pathways that may impact the continuum between putative risk factors and neoplastic outcomes. Much of her research has been related to the molecular epidemiology of breast cancer and elucidation of possible etiologic mechanisms, including the role of hormone metabolites, chemical carcinogens, and oxidative stress. She is also involved in studies of gene-environment interactions and the risk of prostate, lung, and colon cancers.

Maarten C. Bosland

Dr. Bosland is an Associate Professor of Environmental Medicine and Urology at New York University School of Medicine, New York, NY. Born and educated in The Netherlands, Dr. Bosland holds doctoral degrees in Veterinary Medicine (1978) and Experimental Pathology (1989) from Utrecht University, Utrecht, The Netherlands.

Dr. Bosland began his career in 1978 as a research scientist/toxicologic pathologist, Department of Biological Toxicology, Institute CIVO-Toxicology and Nutrition TNO, Zeist, The Netherlands. From 1982 until 1984, he was a visiting scientist at the Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY. He served as a Research Assistant and Professor of Environmental Medicine, Department of Environmental Medicine, New York University Medical Center, New York (1985 to 1987). Returning to The Netherlands in 1985, Dr. Bosland served as an Adjunct Research Scientist, Department of Biological Toxicology, Institute CIVO-Toxicology and Nutrition TNO. Since 1987, Dr. Bosland has served as a faculty member at New York University, where he received tenure in 1993.

Dr. Bosland has authored 70 English publications. His primary areas of research interest are hormonal and prostate carcinogenesis, translational research in prostate cancer chemoprevention using preclinical models and clinical trials, and animal models of prostate cancer.

Ercole L. Cavalieri

Dr. Cavalieri is a Professor in the Eppley Institute for Research in Cancer and Allied Diseases (Omaha, NE) and Director of The Center for Environmental Toxicology at the University of Nebraska Medical Center (Omaha, NE). Born in Milan, Italy, he received a D.Sc. in Chemistry in 1962 from the University of Milan, Milan, Italy. After receiving his degree, he was a postdoctoral fellow at Polytechnic of Zurich, Zurich, Switzerland (1963), where he conducted research in the photochemistry of steroid hormones. He was then a researcher, lecturer, and Assistant Professor in the Department of Chemistry, University of Montreal, Montreal, PQ, Canada, from 1965 to 1968, where he conducted research in organic photochemistry and mechanisms of ozonolysis. Dr. Cavalieri came to the United States in 1968 as a Damon Runyon Postdoctoral Fellow in the laboratory of Nobel laureate Dr. Melvin Calvin, Chemical Biodynamics Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, CA, where he began work on the mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons.

In 1971, Dr. Cavalieri came to the Eppley Institute as an Assistant Professor, where he has continued to work on the mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons. In 1978, he received academic tenure; in 1981, he was promoted to Full Professor. From 1985 to 1986, Dr. Cavalieri served as a University of Nebraska Medical Center Honor Lecturer of the Mid-America State Universities Association. In 1994, he received the Outstanding Research and Creative Activity Award from the University of Nebraska. Dr. Cavalieri is the author of eight book chapters and 150 scientific publications, mainly in the area of the mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons and estrogens.

His major research interests include mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons (with special emphasis on the mechanism of tumor initiation) and mechanisms of tumor initiation by specific metabolites of estrogens (estrogen-3,4-quinones) and their role in the initiation of human cancer.

Cyrus R. Creveling

Dr. Creveling recently retired from his position as the Director of the Office of Technology Development at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD. Born in Washington, DC, he received his Ph.D. in Pharmacology from The George Washington University, Washington, DC, in 1962. He worked as a chemist for the National Heart, Lung, and Blood Institute, Bethesda, MD, from 1955 to 1962 before becoming a research associate at Harvard University, Cambridge, MA (1962 to 1964). He also served as an Adjunct Professor for Howard University, Wash-

ington, DC, (Pharmacology, 1964 to 1986) and the Medical College of Virginia, Richmond, VA (1975 to 1990) while working as a chemist in the Bio-organic Chemistry laboratories at NIDDK (1964 to 1998), before becoming the Director in 1990.

He served the Society for Experimental Biology and Medicine as President of the Washington Chapter, 1975 to 1979, and received the Distinguished Lecturer Award, Enzymology, 1980. He served as President of the Washington Academy of Sciences (1998 to 1999), Fellow of the World Society of Arts and Science, and Foundation for Advanced Education in the Sciences (Awards Committee, Chairman) and he serves on the Board of Directors of the National Institutes of Health Alumni Association. He also served on the Editorial Board for the *Journal of Medical Chemistry* in 1975, and the *Journal of Neurodegeneration* in 1998 and was on the Organizing Committee for the International Catecholamine Symposia, numbers 4 through 9.

He has authored 156 papers and 26 book chapters and he was co-editor of the Transmethylation Series, 1978, 1981, 1984, and editor of *The Role of Catechol Quinones in Cellular Toxicity*, 1999. Major research interests include the role of catechol-*O*-methyltransferase in the inactivation of catecholestrogens and catecholamines, the pharmacologic properties of fluorine-substituted biogenic amines, and the interaction of batrachotoxin and other frog toxins with ion channels.

Robert B. Dickson

Dr. Dickson is a Professor of Cell Biology and Pharmacology and Associate Director for Basic Science, Lombardi Cancer Research Center, Georgetown University, Washington, DC. In addition, he serves as Director of their Tumor Biology Ph.D. Program. He received his M.S. and Ph.D. (1980) in Pharmacology from Yale University, New Haven, CT. Dr. Dickson was a Senior Staff Fellow and Investigator at the National Cancer Institute, Bethesda, MD, (1983 to 1988), before moving to Georgetown University as an Associate Professor. In 1994, he was promoted to Full Professor.

Dr. Dickson has authored more than 260 publications and served as an editor of 10 books and many single-topic journal issues on growth factors, breast cancer biology, and pharmacology. He is the editor of *Breast Cancer Research and Treatment* and serves on the Editorial Boards of several journals, which include *The Journal of Steroid Biochemistry* and *Molecular Biology and Life Sciences*. His research interests include estrogen regulation of gene expression, estrogen receptor-mediated processes in normal and cancer cells, and the role of estrogens in breast cancer.

Krystyna Frenkel

Dr. Frenkel is a Professor in the Nelson Institute of Environmental Medicine at the New York School of Medicine, New York, NY. Born in Poland, she received her Ph.D. in Biochemistry/Chemistry of Nucleic Acids from New York University in 1974. She was a Research Scientist in the Department of Organic Chemistry at Warsaw University, Warsaw, Poland, from 1964 to 1968, before returning as an Assistant Research Scientist to the Department of Environmental Medicine at the New York University Medical Center (1969 to 1974). Dr. Frenkel has spent the last 22 years at New York University Medical Center, where she is currently a member of the Kaplan Comprehensive Cancer Center, Director of the Laboratory of Oxidative Mechanisms in Carcinogenesis, Department of Environmental Medicine, and a

Professor in the departments of Environmental Medicine and Pathology.

Dr. Frenkel has authored 85 publications, has received the Young Environmental Scientist Award (1979 to 1981), and has served as a council member for the Oxygen Society (1994 to 1998). She has been a Member of the National Institutes of Health Reserve since 1995, and she has also served on the editorial boards of *Mutation Research-DNA Repair* since 1995 and *Women and Cancer* since 1998. She has served on the External Advisory Board for the Clinical Nutrition Research Unit at the Memorial Sloan-Kettering, New York, NY, since 1996 and for the Strang Cancer Prevention Center, New York, NY, since 1998.

Dr. Frenkel's major research interests include the following: 1) mechanisms of DNA modification by chemical and physical carcinogens, in relation to initiation and promotion; 2) genetic effects of DNA modification; 3) role of oxidant formation and oxidative DNA damage in diseases; 4) role of environmental and occupational pollutants in genetic damage; 5) chemoprevention; and 6) biomarkers of oxidative stress and cancer in humans.

Montserrat Garcia-Closas

Dr. Garcia-Closas is a Tenure-track Investigator in the Division of Cancer Epidemiology and Genetics (DCEG) of the National Cancer Institute (NCI), Bethesda, MD. Born in Barcelona, Spain, she received her Doctorate in Public Health from the Harvard School of Public Health, Boston, MA, in 1996. She began her career with DCEG, NCI, as a Fogarty Visiting Fellow (1996 to 1997) and Fogarty Visiting Associate (1997 to 1999) before being promoted to a Tenure-track Investigator in 1999. Author of 16 publications, she has received scholarships from C.I.R.I.T. (Interdepartmental Commission for Research and Technologic Innovations), Catalan Government, Barcelona, Spain (1991 to 1992), the Spanish Ministry of Education and Science, Programa Nacional de Becas de Formación de Personal Investigador en el Extranjero, Subprograma de Perfeccionamiento de Doctores y Tecnólogos, Madrid, Spain (1992 to 1994), and the Real Colegio Complutense, Committee on General Scholarships, Harvard University, Cambridge, MA (1994 to 1996), prior to her Visiting Fellow Award from the National Institutes of Health Fogarty International Center in 1996.

Major research interests of Dr. Garcia-Closas include molecular epidemiology studies of genetic susceptibility to cancer, methodologic work on sample size needs and the impact of exposure and biomarker misclassification in studies of gene-environment interactions, and new approaches for the collection and processing of biologic samples for molecular epidemiology studies.

Susan Elizabeth Hankinson

Dr. Hankinson is an Associate Professor in the Schools of Medicine and Public Health at Harvard University and an Associate Epidemiologist at Brigham and Women's Hospital, Boston, MA. She received her D.Sc. in Epidemiology from Harvard University's School of Public Health in 1992.

Dr. Hankinson is a recipient of a National Institute of Environmental Health Sciences National Research Service Award (1989 to 1992) and a 1996 to 2000 United States Army Medical Research and Materiel Command Breast Cancer Research Program Career Development Award. She has authored 47 papers and 11 reviews.

Dr. Hankinson is interested in the relationships between endogenous hormone levels and risk of cancer, including breast, ovarian, and endometrial cancers. A recent focus has been on insulin-like growth factor-1, steroid hormones, and prolactin in relation to breast cancer risk. She is also interested in methodologic issues related to the measurement of hormone levels in both premenopausal and postmenopausal women.

Elizabeth A. Hart

Born in Moulton, AL, Ms. Hart is President and CEO of Hart International, Dallas, TX. A graduate of Brigham and Women's Hospital School of Nursing in Boston, MA, she also holds a B.A. in Psychology from George Washington University, Washington, DC. She has served in numerous leadership positions, including chairman of the board of the Susan G. Komen Breast Cancer Foundation and chairman of the National Cancer Institute (NCI)/Komen Breast Cancer Leadership Summits. A member of the Department of Defense's Breast Cancer Research Program Integration Panel from 1993 to 1996, she served on several subcommittees and was a member of the Technical Program Committee for the Department of Defense's "Era of Hope" meeting in breast cancer research in November 1997. Her testimony before a blue-ribbon panel convened by the Institute of Medicine in 1993 helped shape the beginnings of the Department of Defense Breast Cancer Research Program. She also testified before the President's Special Commission on Breast Cancer, the Food and Drug Administration's Oncological Drug Advisory Committee, and NCI's Board of Scientific Counselors and was appointed in 1997 to the newly formed National Cancer Policy Board. A passionate educator and advocate on breast cancer issues, she was the creative spirit and executive producer of a public television film, "For Women's Lives—Dialogues on Breast Cancer," which was aired in 1995 to 1996 and a finalist in the American Medical Association's International Film Competition. As an invited participant in many national symposia and panels, she currently consults and serves as a breast cancer advocate to several research groups. Ms. Hart has had multiple family members diagnosed with breast cancer and other cancers, including lung, kidney, and prostate cancers. An active public speaker and fundraiser for cancer research, she has directed regional and national campaigns that have raised millions annually.

Shuk-mei Ho

Dr. Ho is a Professor in the Division of Urology, Department of Surgery and Cell Biology, University of Massachusetts Medical School, Worcester, MA. She received a Ph.D. in zoology from the University of Hong Kong in 1978. She joined the Department of Biology, Tufts University, Boston, MA, in 1981 as an Assistant Professor, rising to the rank of Full Professor in 1995. She moved to the University of Massachusetts Medical School in 1999.

Dr. Ho chaired the Gordon Conference on Hormonal Carcinogenesis in 1999. She has published more than 60 scientific articles. Her major research interests include mechanisms of hormonal carcinogenesis, animal models for prostate cancer, the role of androgens and estrogens in prostate carcinogenesis, and hormone receptor-mediated events in prostate carcinogenesis.

Colin R. Jefcoate

Dr. Jefcoate is a Professor in the Department of Pharmacology and Director of the Environmental Toxicology Center, Uni-

versity of Wisconsin, Madison, WI. Dr. Jefcoate received his Ph.D. in Chemistry from Oxford University, Oxford, U.K., in 1966. From 1966 to 1969, he was a NATO Fellow, conducting research at the University of Basel, Basel, Switzerland, and Cornell University, Ithaca, NY. He then was an Medical Research Council Fellow at Edinburgh University, Edinburgh, Scotland, from 1969 to 1972. In 1973, he joined the Department of Pharmacology, University of Wisconsin, as an Assistant Professor, rising to the rank of Professor in 1982.

He has published more than 135 scientific articles. His major research interests include induction and modulation of cytochromes P450 and the metabolism of estrogens, polycyclic aromatic hydrocarbons, and other xenobiotics.

Joachim G. Liehr

Dr. Liehr is the Chief Pharmacologist at the Stehlin Foundation for Cancer Research, Houston, TX. Born in Namslau, Germany, he received his Ph.D. in Synthetic Organic Chemistry from the University of Delaware in 1968. From 1968 to 1969, he served as a Research Chemist for the Space Sciences Laboratory at the University of California, Berkeley, CA, and then as a Research Assistant at the Technische Universität München, München, Germany, from 1970 to 1971. He spent 1 year with Ciba-Geigy, Ltd., in Basel, Switzerland, as a Visiting Research Chemist and 2 years at the Baylor College of Medicine, Houston, TX, as a Visiting Assistant Professor. He returned to Ciba-Geigy in 1974 as a Research Chemist and then joined The University of Texas Medical School (Galveston, TX) where he served in a variety of positions including his current Adjunct Professorship in Pharmacology and Toxicology in Galveston.

Dr. Liehr has authored 148 articles and 17 book chapters and was a featured scientist on the cover of the *International Journal of Oncology* in April 1997. For the past 20 years, his research has been focused on understanding the mechanism of estrogen-induced carcinogenesis in rodent models. He has developed a concept that estrogens may act as complete carcinogens and initiate tumors by metabolic activation and estrogen-induced genetic damage, in addition to their hormonal actions. According to this hypothesis, the carcinogenic process may be completed by hormonal stimuli of estrogens via receptor-mediated processes. The possible induction of human breast cancer by estrogens acting in a dual role as procarcinogens and as hormonal stimulants is presently the focus of Dr. Liehr's research.

David G. Longfellow

Dr. Longfellow was born in Akron, OH, and received his Ph.D. in Biology (Molecular) from The Johns Hopkins University, Baltimore, MD, in 1972. After conducting postdoctoral research as a Damon Runyon Fellow (1972 to 1974), a National Cancer Institute (NCI) Fellow (1974 to 1975), and Biologist (1975 to 1976) in the Laboratory of Biology, Division of Cancer Biology and Diagnosis, NCI, Bethesda, MD, he joined the Division of Cancer Cause and Prevention (DCCP), NCI, as a Biologist in the Carcinogenesis Program in 1976. From 1979 to 1983, he served as Assistant Chief, Chemical and Physical Carcinogenesis Branch (CPCB), Carcinogenesis Extramural Program, DCCP, NCI. In 1983, he became the Acting Chief, CPCB, Division of Cancer Etiology (DCE), NCI. From 1984 to 1995, he served as Chief, CPCB, DCE and, in 1995, also as Acting Special Assistant for Environmental Cancer, Office of the Director.

DCE, NCI. Since 1995, he has been the Chief, CPCB, Division of Cancer Biology, NCI.

As Chief of the CPCB, he is responsible for the scientific administration of more than 300 active and competing research project grants totaling more than \$88 million in Fiscal Year 1998. CPCB is an extramural branch in the largest extramural division of the NCI. Dr. Longfellow maintains research interests in cell biology, toxicology, chemical carcinogenesis, and hormonal carcinogenesis and prevention. His branch initiates, coordinates, evaluates, and maintains an extramural basic and applied research program in chemical, molecular, and physical carcinogenesis and cancer biochemistry. It is also the focal point within the NCI for basic research in chemoprevention with both natural and synthetic chemicals. CPCB is the major focal point in the NCI for environmental toxicology and carcinogenesis. The branch provides liaison activities to other U.S. Government agencies concerned with toxicology, including the Department of Defense, Environmental Protection Agency, Food and Drug Administration, National Institute of Occupational Safety and Health, National Toxicology Program, Occupational Safety and Health Administration, U.S. Air Force, and the U.S. Department of Agriculture.

Donald C. Malins

Dr. Malins is Principal Scientist and Director of the Molecular Epidemiology Program at Pacific Northwest Research Institute in Seattle, WA. Born in Lima, Peru, he received his Ph.D. from the University of Aberdeen, Aberdeen, Scotland, in 1967. Dr. Malins is a member of The University of Washington National Institute of Environmental Health Sciences Center for Ecogenetics and Environmental Health, Seattle, WA, and a Board Member and Senior Scientific Consultant for Biomark, Inc., Boise, ID. He served as a Member of the Breast Cancer Etiology Working Group for the National Action Plan on Breast Cancer (1995 to 1996); as a Scientific Consultant for the U.S. National Oceanic and Atmospheric Administration (1990 to 1992); as a Member of the Great Lakes Science Advisory Board, International Joint Commission (1990 to 1991); as a Senior Scientific Consultant for the U.S. Department of Justice (1989 to 1991); as the Director of the Environmental Biochemistry Program for Pacific Northwest Research Foundation (1986 to 1992); and from 1980 to 1995, as Editor-in-Chief and Founding Editor, *Aquatic Toxicology* (Elsevier Biomedical Press, Amsterdam, The Netherlands).

Dr. Malins' honors include the Arthur S. Fleming nomination (Washington, DC) in 1969 in recognition as being one of "top twenty" scientists under age 40 years in the U.S. Government (sponsored by the U.S. Civil Service Commission and Jaycees of Washington, DC), a Doctor of Science from the University of Aberdeen in 1976 for 20 years of "high distinction" and accomplishment in scientific research, and the U.S. Department of Commerce Gold Medal in 1982 for distinguished scientific contributions. He became a member of the National Academy of Sciences of the USA in 1995.

Author of 200 articles, he has also served as co-editor of "Aquatic Toxicology: Cellular, Biochemical and Molecular Perspectives" (Lewis Publishers, Inc.: Chelsea, MI, 1993), Volumes I-IV, and "Biochemical and Biophysical Perspectives in Marine Biology" (Academic Press: London, U.K., 1974-1978) and as editor of "Effects of Petroleum on Arctic and Subarctic Marine Environments and Organisms," Volumes I-II. Vol. I. Nature and

Fate of Petroleum; Vol. II. Biological Effects (Academic Press: New York, NY, 1977).

His research interests include the following studies in biochemistry: 1) structural damage to DNA in relation to breast, ovarian, and prostate cancers and environmentally induced cancer in aquatic vertebrates; 2) the etiology and prediction of cancer in relation to radical generation via redox cycling of estrogens and xenoestrogens and attendant mutagenic changes in DNA; and 3) the prediction and diagnosis of cancer based on gas chromatographic-mass spectrometric and Fourier-transform infrared spectral models of DNA structure.

Rebecca Raftogianis

Dr. Raftogianis is an Associate Member in the Department of Pharmacology at Fox Chase Cancer Center, Philadelphia, PA. Dr. Raftogianis was born in Hillsboro, NH, and received a Ph.D. in the Department of Pharmaceutics and Pharmaceutical Chemistry at the University of Utah, Salt Lake City, UT, in 1995. Dr. Raftogianis received postdoctoral training in the Department of Pharmacology at the Mayo Clinic, Rochester, MN. Her work there included the cloning of human phenol sulfotransferase genes and the identification of common genetic polymorphisms in those genes. She serves as a Guest Lecturer in Clinical Pharmacology courses at the Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA, and has authored 15 publications. Dr. Raftogianis' interests include the study of the molecular pharmacogenetics of human conjugating and deconjugating reactions. She is particularly interested in identifying the contribution of polymorphisms in human sulfotransferases and glucuronosyltransferases to interindividual variation in the clinical response to drugs.

Eleanor G. Rogan

Dr. Rogan is a Professor in the Eppley Institute for Research in Cancer and Allied Diseases at the University of Nebraska Medical Center, Omaha, NE. Born in Cincinnati, OH, she received her Ph.D. in Biology (Biochemistry) from The Johns Hopkins University, Baltimore, MD, in 1968.

Dr. Rogan's professional appointments include the following: U.S. Public Health Service Predoctoral Fellow, The Johns Hopkins University (1965 to 1968); Lecturer, Department of Biology, Goucher College, Towson, MD (1968 to 1969); and postdoctoral fellow in the Department of Biochemistry (1969 to 1971) and Research Associate, Department of Microbiology, University of Tennessee, Knoxville, TN (1971 to 1973). She joined the Eppley Institute for Research in Cancer, University of Nebraska Medical Center, in 1973 and became Assistant Professor in 1976, Eppley Institute for Research in Cancer and Department of Pharmaceutical Sciences, University of Nebraska Medical Center, and Professor in 1990. She served as Mid-America State Universities Association Honor Lecturer (1988 to 1989).

Dr. Rogan has published more than 140 scientific articles. Her major research interests include mechanisms of tumor initiation by polycyclic aromatic hydrocarbons, conversion of carcinogen-DNA adducts into oncogenic mutations, and the role of endogenous catechol estrogens in the initiation of breast, prostate, and other human cancers.

Deodutta Roy

Dr. Roy is an Associate Professor, Department of Environmental Health Sciences, The University of Alabama, Birmingham.

ham, AL. He received his Ph.D. in Life Sciences (Biochemistry) from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India, in 1984. Following a postdoctoral fellowship (1985 to 1989), he was named an Assistant Professor in the Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX, in 1990. In 1991, he moved to the Department of Environmental Health Sciences, The University of Alabama at Birmingham, where he is also a Scientist in the Comprehensive Cancer Center, and was promoted to Associate Professor in 1994. From 1992 to 1996, he was awarded a Junior Faculty Development Award from the American Cancer Society.

Dr. Roy is the author of more than 70 scientific publications. His major research interests include hormonal carcinogenesis, defects in DNA repair, and estrogen-induced perturbations in genomic stability and DNA repair and their implications in the development of cancer.

Jose Russo

Professor Russo is a Senior Member and Director of the Breast Cancer Research Laboratories of the Fox Chase Cancer Center, Philadelphia, PA. Born in Mendoza, Argentina, his academic career began in 1960 as an Instructor in Pathology at the School of Medical Sciences of the University National of Cuyo, Mendoza, Argentina. After his graduation as Physician and Surgeon in 1967 and upon completion of his medical degree in 1968, he was awarded a Postdoctoral Fellowship by the Argentinean National Council for Research (1968 to 1971). In 1971, he was awarded a fellowship from the Rockefeller Foundation; in 1972, he was appointed Chief of the Experimental Pathology Laboratory at the Michigan Cancer Foundation, Detroit, MI, where he spent the next 20 years. In 1980, he became Member and Chairman of the Department of Pathology at the Michigan Cancer Foundation and Clinical Associate Professor of Pathology at Wayne State University Medical School, Detroit. In 1991, Professor Russo became Chairman of the Department of Pathology at the Fox Chase Cancer Center. In 1995, he became the Director of Breast Cancer Research Laboratories, Fox Chase Cancer Center, establishing a unique program in breast cancer research with basic, translational, and clinical implications.

Dr. Russo is Adjunct Professor of Pathology and Cell Biology at Jefferson Medical School and Adjunct Professor of Pathology and Laboratory Medicine at the University of Pennsylvania Medical School, Philadelphia, PA. He has authored more than 230 publications and four books. He is a member of several editorial boards of scientific journals. Professor Russo has trained more than 40 Ph.D. and M.D. investigators in cancer research.

Dr. Russo's major research interests include cell transformation by chemical carcinogens, hormonal effects on carcinogenesis, hormonally induced differentiation, and prevention of breast cancer.

Richard J. Santen

Dr. Santen is a Professor of Medicine in the Division of Hematology, Oncology, and Endocrinology and Associate Director of the Cancer Center—Clinical Research at the University of Virginia Health Science Center, Charlottesville, VA. Born in Cincinnati, OH, he received his M.D. from the University of Michigan in 1965.

Author of 310 articles, his major research interests include

the following: 1) estrogen control of proliferation of breast cancer, 2) development of inhibitors of aromatase in the treatment of breast cancer, 3) evaluation of aromatase expression in normal breast and breast cancer tissues, and 4) development of strategies to bypass the need for estrogen in survivors of breast cancer.

George M. Stancel

Dr. Stancel is a Professor of Integrative Biology, Pharmacology, and Physiology and Dean of the Graduate School of Biomedical Sciences at The University of Texas Medical School, Houston, TX. He was born in Chicago, IL, and received his Ph.D. in Biochemistry from Michigan State University, Lansing, MI, in 1970. Following postdoctoral research in endocrinology at the University of Illinois, Urbana, IL, from 1970 to 1972, he moved to the Department of Pharmacology, The University of Texas Medical School, as an Assistant Professor. From 1990 to 1996, he served as Chair, Department of Pharmacology, before advancing as Professor to the Department of Integrative Biology, Pharmacology, and Physiology.

Dr. Stancel received a National Institutes of Health Research Career Development Award (1976 to 1981). He is a member of the Editorial Board of *Endocrinology* and serves as Associate Editor of the *Journal of Pharmacology and Experimental Therapeutics*. He has served as a member of the Environmental Protection Agency's Advisory Panel on Endocrine Disruptors and the National Institute of Environmental Health Sciences Environmental Health Sciences Review Committee. He has chaired Gordon Research Conferences on Hormonal Carcinogenesis (1991) and Reproductive Tract Biology (1994). He is the author of more than 200 scientific articles.

His major research interests include estrogen regulation of gene expression, estrogenic control of uterine cell proliferation, mechanism of action of endocrine disruptors, and regulation of endometrial gene expression by estrogen replacement therapy.

Thomas R. Sutter

Dr. Sutter is a Professor and the Feinstone Chair of Excellence in Functional Genomics in the Department of Microbiology and Molecular Cell Science, the University of Memphis, Memphis, TN. He received his Ph.D. in environmental health from the University of Cincinnati College of Medicine, Cincinnati, OH, in 1988. Following postdoctoral research at the Chemical Industry Institute of Toxicology, Research Triangle Park, NC, in 1991, he joined the Department of Environmental Health Sciences, Division of Toxicological Sciences, at The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD, as an Assistant Professor. He served as an Associate Professor until 1999, when he moved to the University of Memphis.

Author of more than 40 publications, he served on the National Center for Environmental Assessment, Advisory Committee for Revision of Dioxin Risk Assessment. His major research interests include metabolism of estrogens and xenobiotic compounds by cytochromes P450, expression and function of cytochrome P450 1B1 in animal models and human cells, and the role of estrogens in human breast cancers and other cancers.

Patricia A. Thompson

Dr. Thompson is an Assistant Professor at The University of Texas M. D. Anderson Cancer Center (MDACC) in the Depart-

ment of Epidemiology, Houston, TX. She was born in Galveston, TX, and received her Ph.D. in Microbiology from The University of Texas Health Science Center, San Antonio, TX, in 1993. From 1996 to 1998, Dr. Thompson was a postdoctoral fellow in the Division of Molecular Epidemiology at the National Center for Toxicological Research (NCTR), Jefferson, AR, where she focused her studies on individual susceptibility to chemical and hormonal carcinogenesis. Following promotion to Staff Fellow in 1998, Dr. Thompson, working in collaboration with her colleagues at NCTR, MDACC, and Genometrix Inc. (The Woodlands, TX), has focused her efforts to develop high throughput DNA microarray-based technologies to assess multiple genetic polymorphisms in relation to disease risk. After returning to Texas to the Department of Epidemiology at the MDACC, she is continuing to pursue the identification of biologic markers, using minimally invasive procedures (e.g., blood, urine, and buccal swab) and high throughput technologies to advance our understanding of individual disease risk and disease outcome.

Richard Weinshilboum

Dr. Weinshilboum is Professor of Pharmacology and Medicine at the Mayo Medical School/Mayo Clinic, Rochester, MN. After receiving both B.A. and M.D. degrees at the University of Kansas, Lawrence, KS, Dr. Weinshilboum was a Resident in Internal Medicine at the Massachusetts General Hospital, Boston, MA. He was also a Pharmacology Research Associate at the National Institutes of Health, Bethesda, MD in the laboratory of Nobel laureate Dr. Julius Axelrod.

Dr. Weinshilboum began his affiliation with the Mayo Medical School and Mayo Clinic in 1972. In the ensuing years, he has served, at various times, as Chief of the Mayo Clinical Pharmacology Unit, Chair of the Department of Pharmacology, Director of Research, Mayo Foundation, and Director for Education, Mayo Foundation. Dr. Weinshilboum was recently appointed to the National Advisory Scientific Council for the National Institute of General Medical Sciences. He has also served as President of the American Society for Clinical Pharmacology and Therapeutics. He has authored more than 200 manuscripts, and his honors include graduating Phi Beta Kappa "with highest distinction," Alpha Omega Alpha membership, and receipt of the American Heart Association Established Investigatorship and a Burroughs Wellcome Scholar in Clinical Pharmacology Award. He was also selected as the Rawls Palmer and Oscar B. Hunter Awards recipient by the American Society for Clinical Pharmacology and Therapeutics.

Dr. Weinshilboum's primary research interests involve the role of inheritance in variation in response to drugs and xenobiotics, including carcinogens—that is, the fields of pharmacogenetics and pharmacogenomics. He has placed special emphasis on the role of conjugation reactions such as methylation and sulfation in genetic variations in the biotransformation of drugs and carcinogens.

Judith Weisz

Dr. Weisz is a Professor in the Division of Maternal and Fetal Medicine, Department of Obstetrics and Gynecology, The Medi-

cal Center, The Pennsylvania State University, Hershey, PA. She received an M.B. B.Chir. (Bachelor of Medicine, Bachelor of Surgery) from Cambridge University, Cambridge, U.K., in 1951. Following fellowships in the Department of Endocrinology, Mount Sinai Hospital, New York, NY (1960 to 1962), and the Training Program in Steroid Biochemistry, The Worcester Foundation for Experimental Biology, Shrewsbury MA, (1962 to 1963), she was a Staff Scientist and Associate Director of The Training Program in the Physiology of Reproduction at The Worcester Foundation (1963 to 1970). In 1972, she joined the Department of Obstetrics and Gynecology at The M. S. Hershey Medical Center, Hershey, PA, as an Associate Professor, rising to the rank of Professor in 1983.

Dr. Weisz has served on the editorial boards of *Endocrinology and Cell Biochemistry and Function* and as an Associate Editor of *Quantitative Cytochemistry, International Review of Cytology*. She has published more than 110 scientific articles. Her major research interests include estrogen metabolism in the human breast and other tissues, mechanisms of induction of cancer by estrogens, and the role of estrogen metabolites in breast cancer.

James D. Yager, Jr.

Dr. Yager is Professor of Toxicology and Director, Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD. He also serves as Director of the National Institute of Environmental Health Sciences-supported training program in Environmental Health Sciences at The Johns Hopkins University.

Dr. Yager received his Ph.D. in Developmental and Cell Biology from the University of Connecticut, Storrs, CT, in 1971 and was a postdoctoral fellow at the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, from 1971 to 1974. In 1974, Dr. Yager joined the faculty at Dartmouth University, Hanover, NH, as an Assistant Professor of Biology; in 1976, he transferred to the Department of Pathology in the Dartmouth Medical School. In 1981, he became an Associate Professor of Environmental Medicine at New York University, New York, NY; in 1983, he returned to Dartmouth to become an Associate Professor in the Department of Anatomy and Associate Director for Basic Science in the Norris Cotton Cancer Center. He was promoted to Professor in 1986. Dr. Yager was interim chair of the Department of Pharmacology and Toxicology, Dartmouth University, from 1987 to 1989, when he moved to his current position at The Johns Hopkins University.

Dr. Yager has published more than 70 scientific articles and is a member of the editorial board of the *Journal of Environmental Pathology and Toxicology* and *Oncology*. His main research is aimed at understanding the mechanisms of estrogen carcinogenesis, with a focus on estrogen metabolites in breast and ovarian cancer, genetic susceptibility to breast and ovarian cancers through polymorphisms in genes involved in estrogen phase I and II biotransformation, and mechanisms of estrogen (ethinyl estradiol) promotion of hepatocarcinogenesis.

Introductory Remarks

David G. Longfellow

The International Symposium was sponsored by the Division of Cancer Biology (DCB) of the National Cancer Institute (NCI). The organizers of the symposium are also pleased to acknowledge the generous support of the National Institutes of Health Office of Research on Women's Health and the Susan G. Komen Breast Cancer Foundation. Special gratitude is expressed for the invaluable assistance of Karen R. Grotzinger, NCI, for meeting coordination. The organizers also thank Mrs. Fran Oscar and her staff at Palladian Partners for their excellent logistical and on-site support. The preparation of this monograph would not have been possible without the dedicated effort of the monograph editors, Drs. Ercole L. Cavalieri and Eleanor G. Rogan, of the Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, together with the expert technical assistance of Mr. Harry W. Bullerdiek.

The March Symposium was the intellectual outcome of a new NCI extramural initiative, a focus group on estrogen carcinogenesis. This focus group is known as the "Cancer Cube." The term "Cube" is derived from the essential elements on which this focus group is based: Complementary, Collaborative, Coalition, which is "C" to the third power (C^3). The "Cancer Cube" that is currently being piloted by the Chemical and Physical Carcinogenesis Branch in the Division of Cancer Biology is a new paradigm for NCI to facilitate cancer research. The Cube is composed of active researchers who have, by mutual agreement, joined forces in an ongoing effort to better focus on a scientific question or problem in cancer that is of interest to the NCI and the National Cancer Program. The goal that this group has established for themselves is to achieve synergism in the overall effort by enhancing the individual research of the members through scientific collaborations and sharing of resources, techniques, and data.

Cancer Cubes can be thought of as "building blocks" to new understanding. The driving force behind the development of this initiative was NCI's desire to find innovative approaches to facilitate progress, the recognition that a critical mass of collaborating scientists is sometimes needed to achieve momentum, and the recognition in this first Cube activity that maverick viewpoints were beginning to converge and complement each other. A frequent recommendation from workshops in various areas of cancer research is that multidisciplinary expertise is needed to address the unanswered questions. However, funding mechanisms typically used to address such needs are cost-intensive and face considerable obstacles in review to overcome the initial inertia of formation. Once funded, these grants must compete with other large-dollar initiatives to maintain continuity.

The C^3 , on the other hand, is not a funding mechanism. It recognizes that active investigators are usually on "soft" money and, as a matter of course, will obtain research project grants to pursue their scientific interests. The vast majority do this through the R01 mechanism. The C^3 seeks to allow scientific interests and not the funding mechanism (e.g., P01, P30, or U01) to drive the collaborative process. The only initial expense is in

bringing the interested parties together to explore their interest in collaborating and then meeting on a regular basis (such as twice a year) to review progress and design new initiatives. With time, it is recognized that the activities of a Cube will increase the demand on core resources as the utilization of reagents, animals, and the like expands. This need can initially be addressed through administrative supplements. Eventually, it is envisioned that a shared resource mechanism (e.g., an R24) could serve to provide free-standing cores for the collaborative efforts of the Cube.

The size of a Cube is not artificially limited by budget constraints but by human dynamics of interaction. The limitation in participants is regulated more by practical issues of discussion and interaction. This number is probably on the order of less than 20 participants. All members are participants in the intellectual process and sharing, but there is no need to collaborate with all of the players at the same time. This distinguishes a Cube from the dynamics of a program project grant (P01) where there would typically be five to six investigators engaged around a central topic. A group of 18–20 researchers provides a richness of diversity in discipline and training and more important, in a viewpoint that one doesn't usually find in the existing funding mechanisms. Highly desired multi-institutional collaborations are easily facilitated, in effect creating an "institute without walls."

Measures of success for an initiative like the C^3 will perhaps vary with the goals set, but some quantifiable measures might include evidence of the following: collaborative publications, new collaborative projects, generation of new concepts, movement of the field forward, new insights into the mechanisms of cancer, translational progress from experimental design to clinical application, and prevention/intervention strategies.

The concept for the first C^3 was developed by an NCI scientific program director, Dr. David Longfellow, who contacted two investigators who were already collaborating and who were committed to making progress in the field of estrogen carcinogenesis. The remaining potential participants were identified and contacted initially by those two investigators. A major consideration was to identify investigators who would bring to the group the range of needed expertise. This Cube began with a roster of 17 members and has since added a member from the breast cancer advocacy community. In its early meeting, the C^3 selected three co-chairs and a central question was developed as a focus and a reachable goal for the group's endeavors. The C^3 decided to meet twice a year to reassess and fine-tune their research.

The research question that the C^3 chose to address was: Do estrogenic compounds induce genotoxic events leading to can-

Affiliation of author: Division of Cancer Biology, National Cancer Institute, Bethesda, MD.

Correspondence to: David G. Longfellow, Ph.D., National Institutes of Health, 6006 Executive Blvd., Suite 220, MSC 7055, Bethesda, MD 20892-7055 (e-mail: dl585@nih.gov).

See "Note" following the text.

cer? Their specific focus is to raise the awareness and understanding of nonreceptor-mediated modes of action. They hypothesize that estrogen tumorigenesis requires the metabolic activation of estrogens to reactive intermediates. Tumors would develop from cells genetically damaged by specific estrogen metabolites and proliferating in response to estrogen receptor-mediated stimuli.

By the fourth meeting of the C³, plans were consolidated to develop a major international symposium. It was envisioned that this meeting would set the stage to develop a more holistic understanding of the role of estrogens in carcinogenesis both through traditional aspects of estrogen receptor binding but also through nonreceptor-mediated mechanisms. World experts were invited to participate. Topics included metabolic activation of estrogens to carcinogenic forms, deactivation of carcinogenic metabolites, and the role of estrogen receptor-mediated processes in tumor induction. One of the goals of the symposium was to provide attendees with an overview of the direction of research on estrogen-induced cancer. Another goal was to identify biomarkers that can be useful in studies of cancer risk among humans and in the future development of preventive strategies. The symposium was very well received by an audience of approximately 160 participants. The 2-day meeting concluded with a panel perspective from the advocacy community with the major organizations in breast and prostate cancers. A preview of the actual meeting, which appeared in the March 13, 1998, issue of *Science*, entitled "New role of estrogen in cancer?", was based on extensive background interviews with a number of the major speakers.

This monograph seeks to tell the story of estrogen research to date and can serve as a resource for both the scientific and lay communities. It will also serve as a record of the scientific progress to date of the Cancer Cube Focus Group on Estrogen Carcinogenesis.

In summary, the first C³ has demonstrated the success of this approach to stimulating new understandings in estrogen carcinogenesis in particular but indeed for cancer etiology and prevention in general. C³ members are publishing together; submitting joint grant applications; visiting and lecturing at each other's institutions; establishing resource, technology, and tissue networks; and strengthening the body of literature in this area of research through their complementary efforts.

To provide opportunities for other researchers to experience similar collaborative success, NCI has made available \$1.125 million per year to the DCB to promote similar collaborative research efforts. In response, the DCB has announced a new initiative entitled Activities to Promote Research Collaborations (APRC), the purpose of which is to promote and facilitate research collaborations in basic cancer research. Limited supplemental support is available for meetings or workshops and for grant-related research activities (e.g., consortia) to establish focused scientific research collaborations in novel and promising areas. To date, four rounds of submission and review have occurred (two in FY98 and two in FY99), resulting in 17 consortia APRC awards totaling \$1.574 million and six workshop APRC awards totaling \$134 000. Three subgroups of the C³ have successfully competed for consortia awards under this initiative.

NOTE

Editor's note: This monograph is based on an International Symposium on "Estrogens as Endogenous Carcinogens in the Breast and Prostate," which was held at the Westfields Conference Center in Chantilly, VA, on March 16 and 17, 1998. It is not in the strictest sense a "proceedings" of that symposium, but the editors of this monograph have sought to enhance the presented materials and to integrate them into a text that will enable greater readability. To that end, the chairpersons of each of the sessions of the symposium have also served as editors of their respective sessions to integrate the contributed manuscripts into chapters for this monograph.

Symposium Overview

Richard J. Santen

My role today is to preview the concepts that will be presented over the course of this meeting and to provide a framework for integration. Estradiol (E_2) can potentially act to mediate carcinogenesis via two separate pathways. One pathway involves receptor-mediated stimulation of biologic events, and the other involves the metabolism of E_2 to compounds, resulting in DNA damage and mutations. As Fig. 1 shows, E_2 binds to its receptor and initiates transcription of genes involved in cellular proliferation. The frequency of genetic mutations parallels the increase in the number of cell divisions. In addition, the time available for DNA repair diminishes. This pathway is generally believed to be the one responsible for estrogen-induced carcinogenesis. Understanding of the physiology of receptor-mediated transcription has increased substantially during the last several years. A second estrogen receptor, the β -receptor, has been described recently and studied intensively. Receptor variants occur; co-activators, corepressors, and integrator proteins modulate estrogen-induced transcription and responsiveness to both estrogens and antiestrogens. These form the subject of several of the talks that review this rapidly advancing field (*see* Chapter 8).

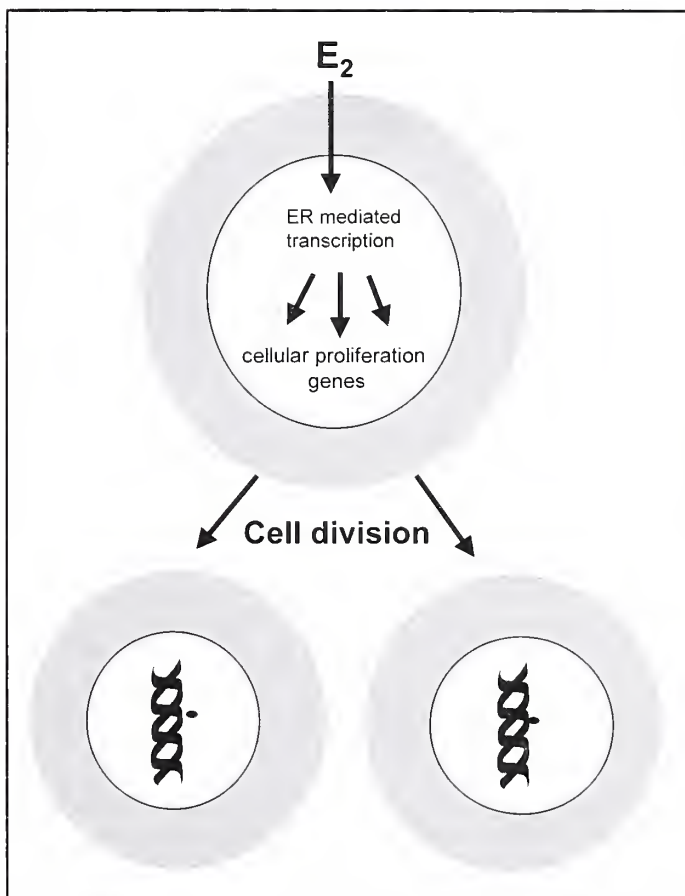


Fig. 1. Estradiol (E_2)-induced carcinogenesis: Estrogen receptor (ER)-mediated transcription.

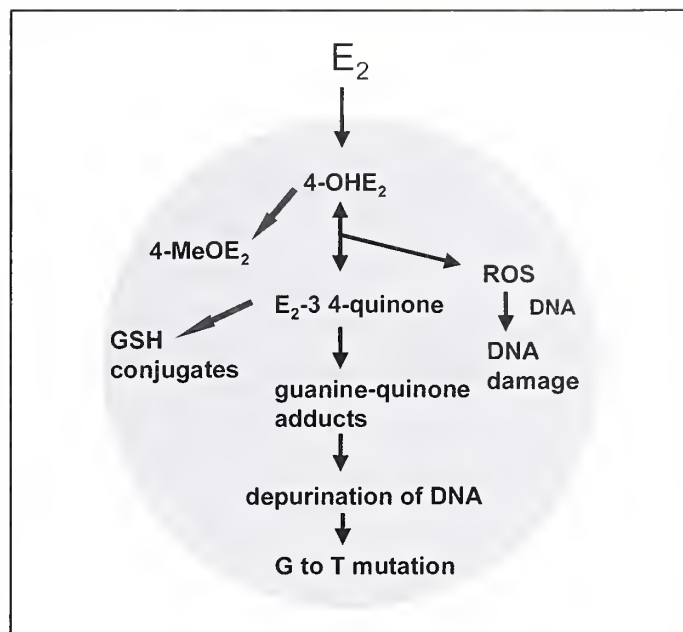


Fig. 2. Estradiol (E_2)-induced carcinogenesis: E_2 metabolism, DNA damage and mutations. GSH = glutathione; 4-MeOE₂ = 4-methoxyestradiol; ROS = reactive oxygen species.

A second pathway (Fig. 2) has been proposed as a mediator of estrogen-induced carcinogenesis. This pathway involves the metabolism of E_2 to the catechol estrogen 4-hydroxyestradiol (4-OHE₂) and then to a further oxidized metabolite, E_2 -3,4-quinone (*see* Chapters 4 and 5). This metabolite can bind to either guanine or adenine in DNA to form guanine-quinone or adenine-quinone adducts. (Only the guanine-quinone adducts are shown in Fig. 2.) Reaction of the quinone with guanine or adenine destabilizes the glycosidic bond, leading to the depurination of the DNA. When the depurinated DNA replicates, both G to T and A to T point mutations can occur.

The metabolism of 4-OHE₂ is reversible through a quinone reductase enzyme. Of interest, this enzyme can be stimulated by tamoxifen. Oxidoreduction between catechol estrogens, semiquinones, and quinones generates reactive oxygen species that damage DNA extensively. Two major pathways are protective of this potentially harmful oxidative metabolic sequence. One involves the catechol-*O*-methyltransferase enzyme, which converts 4-OHE₂ to 4-methoxyestradiol; the other neutralizes E_2 -3,4-quinone by conjugation with glutathione. Alterations of these detoxification steps could enhance or reduce the incidence of breast cancer, a topic to be discussed later (*see* Chapter 6).

Consideration of these two separate pathways does not imply that each may act exclusively of the other (Fig. 3). It is considered likely that these two mechanisms work in either an additive or a synergistic manner to mediate carcinogenesis. The mutations induced specifically by depurinating adducts and generally

Correspondence to: Richard J. Santen, M.D., Division of Hematology, Oncology, and Endocrinology, P.O. Box 334, Cancer Center-Rm. 4023, University of Virginia Health Science Center, Charlottesville, VA 22908 (e-mail: rjs5y@virginia.edu).

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by oxidative DNA damage would be propagated by the genomic effects of E_2 on cellular proliferation.

The plausibility that the metabolic pathway leading to DNA adducts is biologically significant has been questioned. The primary basis for this critique is that insufficient concentrations of E_2 are present in tissue to allow accumulation of biologically meaningful amounts of critical metabolites. However, recent observations suggest that breast tissue can synthesize E_2 *in situ* (see Chapter 5). Under these circumstances, much more E_2 would be present in tissue than would be predicted from plasma concentrations. The rate-limiting step in estrogen biosynthesis is aromatase, a member of the cytochrome P450 class of enzymes (Fig. 4). This enzyme converts androstenedione to E_2 . Breast tissue contains 17 β -oxidoreductase, the enzyme that continuously interconverts

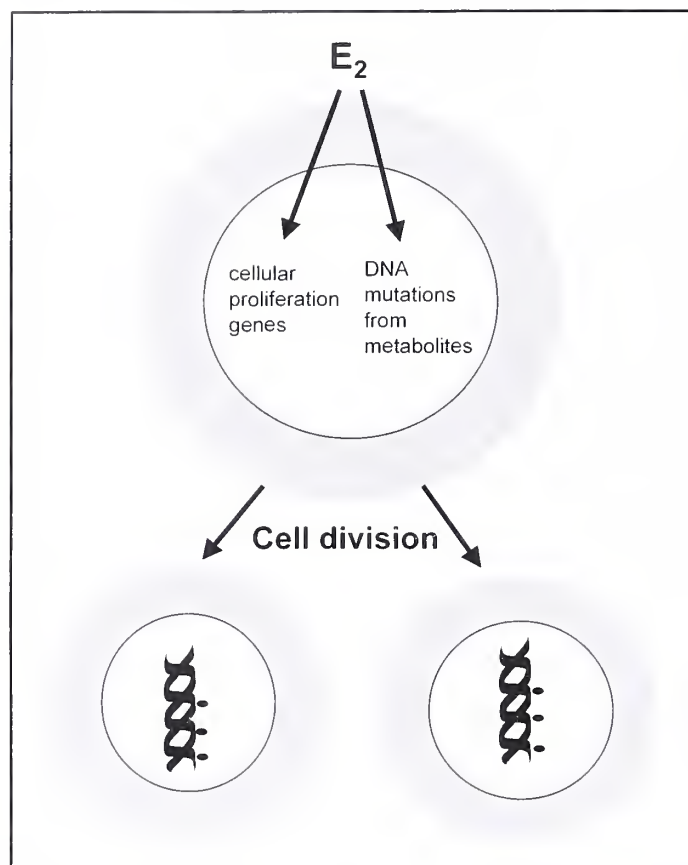


Fig. 3. Estradiol (E_2)-induced carcinogenesis: additive or synergistic interactions.

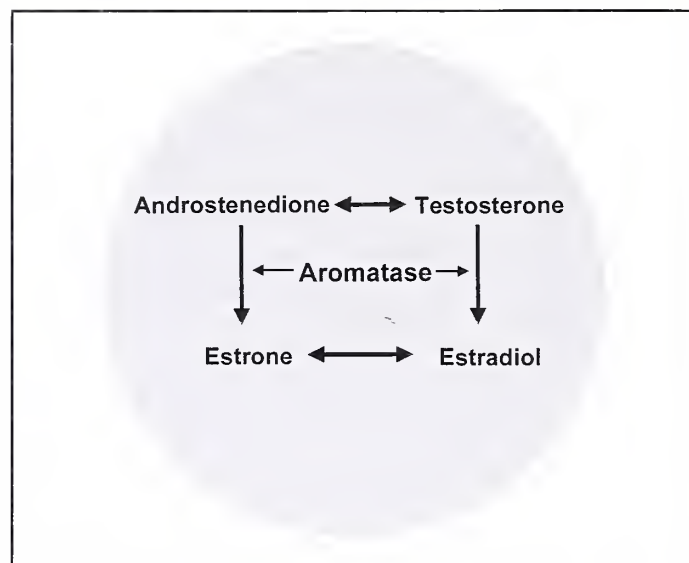


Fig. 4. Estradiol-induced carcinogenesis: enzyme overexpression.

estrone and E_2 . Overexpression of aromatase in the breast would substantially increase tissue E_2 levels. Several animal models of aromatase overexpression and breast cancer have been described, which support the possibility of a role for this enzyme in carcinogenesis. Furthermore, overexpression of cytochrome P450 1B1, which converts E_2 to 4-OHE $_2$, could also result in accumulation of higher amounts of genotoxic E_2 metabolites (see Chapter 5).

Several presentations will address issues of molecular epidemiology suggested by these pathways (see Chapters 7 and 9). For example, population studies may demonstrate that overproduction of key enzymes in E_2 synthesis and/or its metabolism is associated with an increased incidence of breast cancer. Underproduction of detoxifying enzymes could have the same associations.

The presentations to follow will focus on each of the key biologic mechanisms described above. The presenters will integrate the individual steps and pathways and utilize a common pathway overview schemata to focus attention on the relative place of each step in the overall metabolic process.

NOTE

Editor's note: Dr. Santen is a consultant with Eli Lilly and Co. (Evansville, IN) and is conducting research with Novartis Pharmaceuticals Corporation (East Hanover, NJ).

Chapter 1: Developmental, Cellular, and Molecular Basis of Human Breast Cancer

Jose Russo, Yun-Fu Hu, Xiaoqi Yang, Irma H. Russo

Breast cancer, which is the most common neoplastic disease in females and accounts for up to one third of all new cases of women's cancer in North America, continues to rise in incidence. In addition, the mortality caused by this disease has remained almost unchanged for the past 5 decades, becoming only second to lung cancer as a cause of cancer-related death. The failure in eradicating this disease is largely due to the lack of identification of a specific etiologic agent, the precise time of initiation, and the molecular mechanisms responsible for cancer initiation and progression. Despite the numerous uncertainties surrounding the origin of cancer, there is substantial evidence that breast cancer risk relates to endocrinologic and reproductive factors. The development of breast cancer strongly depends on the ovary and on endocrine conditions modulated by ovarian function, such as early menarche, late menopause, and parity. However, the specific hormone or hormone combinations responsible for cancer initiation have not been identified, and their role as protective or risk factors is still incompletely understood. A highly significant female hormone is estrogen, which is involved in the development of a variety of cancers, but it is still unclear whether estrogens are carcinogenic to the human breast. An understanding of whether estrogens cause mutations, and, if so, whether they act through hormonal effects activated by receptor binding, cytochrome P450-mediated metabolic activation, or compromise the DNA repair system, is essential for determining whether this steroid hormone is involved in the initiation or progression of breast cancer. This knowledge has to be based on a multidisciplinary approach encompassing studies of the development of the breast, influence of hormones on the differentiation of individual structures, and their interrelations in the pathogenesis of breast cancer. The analysis of the mechanisms involved would require confirmation in the adequate *in vitro* models and determination of the role played by genomic alterations in both cancer initiation and progression. [J Natl Cancer Inst Monogr 2000;27:17-37]

Breast cancer accounts for up to one third of all new cases of women's cancer in North America, representing the most common neoplastic disease in the female (1). The incidence rates of this disease have been approximately level during the past decade, and breast cancer mortality is declining in the United States and in certain industrialized areas. However, these favorable trends have not been observed in nations with lower breast cancer incidence, and societies traditionally known to have low breast cancer incidence are experiencing an increase in both incidence and mortality (1,2). Even though the U.S. mortality rates in females decreased an average of approximately 1.7% per year from 1990 through 1995, breast cancer retains the first place as a cause of cancer-related death in nonsmoking women (1,2).

The failure in eradicating this disease is largely due to the lack of identification of a specific etiologic agent, the precise time of initiation, and the molecular mechanisms responsible for cancer initiation and progression. Despite the numerous uncertainties surrounding the origin of cancer, intensive epidemiologic, clinical, and genetic studies have identified a number of biologic and social traits as risk factors associated with breast cancer (2-5). Principal among them are the evidence of BRCA1 and BRCA2 susceptibility genes; familial history of cancer in the breast, ovary, or endometrium; individual history of breast diseases; advanced age; higher socioeconomic status; excess ionizing radiation exposure; tallness in adult life; consumption of alcohol; and a variety of endocrinologic and reproductive factors (2-5). These latter factors include early onset of menstruation, nulliparity or delayed first childbirth, short duration of breast-feeding, late menopause, postmenopausal obesity, extended use of oral contraceptives, and prolonged estrogen replacement therapy (2-5).

Among the hormonal influences, a major role has been attributed to the unopposed exposure to elevated levels of estrogens (6), as has been indicated for a variety of female cancers, namely vaginal, hepatic, and cervical carcinomas (7-12). However, the mechanisms through which this phenomenon occurs have not been completely understood (6). In fact, it is still unclear whether estrogens are carcinogenic to the human breast. Most of the current understanding of the carcinogenicity of estrogens is based on clinical observations of a greater risk of endometrial hyperplasia and neoplasia associated with estrogen supplementation (10-12) and experimental data (13-15). At least three mechanisms are considered to be responsible for the carcinogenicity of estrogens; the most widely recognized is the receptor-mediated hormonal activity, which is generally related to stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damage leading to carcinogenesis (16,17). The second mechanism is the cytochrome P450-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates (18-43), and a third mechanism is postulated to compromise the DNA repair system, resulting in the accumulation of lesions in the genome essential to estrogen-induced tumorigenesis (13).

Disappointingly, the molecular mechanisms underlying the development of breast cancer in general, and estrogen-associated breast carcinogenesis in particular, are not completely understood. It is generally believed that the initiation of breast cancer results from uncontrolled cellular proliferation and/or aberrant programmed cell death or apoptosis as a consequence of cumu-

Affiliation of authors: Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA

Correspondence to: Jose Russo, M.D., Breast Cancer Research Laboratory, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA (e-mail: J_russo@fccc.edu).

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lative genetic damages that lead to genetic alterations that result in activation of proto-oncogenes and inactivation of tumor suppressor genes (44,45). Genetic alterations, in turn, can be inherited as germline mutations or acquired as somatic mutations. These latter ones might occur as a result of exposure to environmental carcinogens, either physical (e.g., excess ionizing radiation), chemical (e.g., polycyclic hydrocarbons or nitrosoureas), and/or biologic (e.g., viruses) (5). The classic two-stage animal model of chemical carcinogenesis has constituted the basis for the generally accepted conclusions that the altered genotype of an initiated cell is irreversible and that the expression of transformed phenotypes requires further genetic or epigenetic changes (46). Tumor progression, the second stage of carcinogenesis in this model, involves exclusively epigenetic changes that are considered to be reversible (46). It remains to be determined whether this is true in breast cancer. The mechanism through which endocrinologic factors, such as hormone replacement therapy, influence cancer initiation and progression in women has not been clarified as yet. The development of breast cancer entails multiple events; unfortunately, the two main factors elucidated in the experimental animal model, the causative agent and the time of initiation, are unknown in the human population (47,48). The elucidation of whether estrogens act as endogenous carcinogenic agents requires a better understanding of the normal development of the breast under the influence of physiologic conditions, which in turn is important for understanding the pathogenetic pathway leading to preneoplastic lesions and cancer. The analysis of pure populations of human breast epithelial cells (HBECs) at various stages en route to malignancy would be the direct approach to understanding the cellular and molecular processes of breast carcinogenesis (49). However, it has been extremely difficult to establish primary cultures of HBECs from breast lesions representing various stages of neoplastic progression, and no cell lines at the intermediate stages of neoplastic transformation are available for mechanistic studies (49). It is expected that an *in vitro* system of HBECs that can reproduce the main steps of the *in vivo* situation, such as cell immortalization and transformation, would constitute an adequate tool for determining what genomic changes are important in the initiation and progression of the neoplastic process (50–62). It is, therefore, predicted that an *in vitro* system of this nature will allow us to test whether estrogens are endogenous carcinogens and whether they play an essential role in the initiation and/or the promotional phase of breast cancer.

DEVELOPMENTAL PATTERN OF THE HUMAN BREAST FROM ADOLESCENCE TO MATURITY

The development of the breast, which is rigorously controlled by the ovary, can be defined by several parameters, such as its external appearance, total area, volume, degree of branching, number of structures present in the mammary gland, and degree of differentiation of individual structures, i.e., lobules and alveoli (63–68).

The breast undergoes changes that are progressive from birth to early childhood, becoming striking at puberty. The adolescent period begins with the first signs of sexual change at puberty, which in American females sets in between the ages of 10 and 12 years, and terminates with sexual maturity (64–66). Although puberty is often considered to be the point of initiation of ovarian function, the development of the ovary is a gradual process that depends on pituitary gonadotropins. Receptors for the pituitary

luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are present in the ovary even during the infantile period, when they stimulate the secretion of androgens after binding to and activating their respective receptors (69). FSH and LH interact with growth hormone (GH) and prolactin in modulating ovarian steroidogenesis, a function that is also influenced by epinephrine, secreted by the adrenal medulla. The release of FSH is, in turn, modulated by inhibin and activin, glycoprotein hormones secreted by the ovary (69).

The mammary ductal tree undergoes progressive elongation and branching during childhood and puberty. These processes are positively regulated by GH, although its exact mechanism of action is unclear. GH directly stimulates ductal growth in hypophysectomized–ovariectomized rats, and it might act as well through its local mediator, the insulin-like growth factor I. Normal ductal development, however, requires the presence of estrogen and progesterone, the two ovarian steroid hormones that act on the mammary gland through their respective receptors. As puberty approaches, the rudimentary mamma begins to show growth activity both in the glandular tissue and in the surrounding stroma. Glandular increase is due to the growth and division of small bundles of primary ducts originated during intrauterine life from invaginations of the superficial ectoderm (63,65–67). The ducts grow and divide through a combination of dichotomous and sympodial branching, forming at the distal epithelial–stromal boundary a club-shaped terminal end bud. Each terminal end bud bifurcates into two smaller ductules or alveolar buds (67,70). The term alveolar bud applies to those structures that appear morphologically more developed than the terminal end bud. With further branching, alveolar buds become smaller and more numerous, and then they are called ductules. When an average of 11 alveolar buds/ductules cluster around a terminal duct, they form the lobule type 1 (Lob 1) or virginal lobule (Fig. 1) (67). Terminal ducts and ductules are lined by a two-layered epithelium, whereas terminal end buds in the human fetus are lined by an epithelium composed of up to four layers of cells. Lobule formation in the female breast occurs within 1–2 years after onset of the first menstrual period. Afterward, the ulterior development of the gland varies greatly from woman to woman. Full differentiation of the mammary gland is a gradual process



Fig. 1. Whole mount preparation of breast tissue of an 18-year-old nulliparous woman showing lobule 1. Toluidine blue, $\times 25$.

taking many years, and it can be assumed that in all women in whom pregnancy did not supervene, it was never attained (63).

The normal breast tissue of adult women contains two other identifiable types of lobules in addition to the Lob 1 described above. These are designated lobule type 2 (Lob 2) and type 3 (Lob 3) (Figs. 2 and 3). The transition from Lob 1 to Lob 2 and of these to Lob 3 is a gradual process of sprouting of new ductules, which increase in number from approximately 11 in Lob 1 to 47 and 80 in Lob 2 and Lob 3, respectively (Fig. 3, Table 1). As the number of ductules increases, so does the size of the lobules, even though individual ductules appear reduced in size (Table 1) (63,68).

The breast of nulliparous women contains more undifferentiated structures, such as terminal ducts and Lob 1, although Lob 2 and Lob 3 are occasionally observed. These characteristics are not influenced by age or menopausal status (Fig. 4). In parous premenopausal women, the predominant structure is the most differentiated Lob 3, whose number peaks during the early reproductive years. They start to decrease after the fourth decade of life, as the proportion of Lob 1 increases, and, when menopause sets in, they reach the same values observed in nulliparous women (Fig. 4). In the breast of nulliparous women, the Lob 2 is present in moderate numbers during the early years, sharply decreasing after age 23 years, whereas the number of Lob 1 remains significantly higher. This observation suggests that a certain percentage of Lob 1 might have progressed to Lob 2, but the number of Lob 2 progressing to Lob 3 is significantly lower in nulliparous women as compared with parous women. In the case of parous women, it is interesting to note that a history of parity between the ages of 14 and 20 years is associated with a significant increase in the number of Lob 3 that remains present as the predominant structure until the age of 40 years, the time at which a decrease in the number of Lob 3 occurs, probably because of their involution to predominantly Lob 1 (Fig. 4) (63,68).

HORMONAL INFLUENCES ON THE DEVELOPMENT OF THE BREAST

The breast is a hormone-responsive organ by excellence. Its development is influenced by a myriad of hormones and growth



Fig. 2. Whole mount preparation of human breast tissue of a 24-year-old nulliparous woman showing lobule 2. Toluidine blue, $\times 25$.

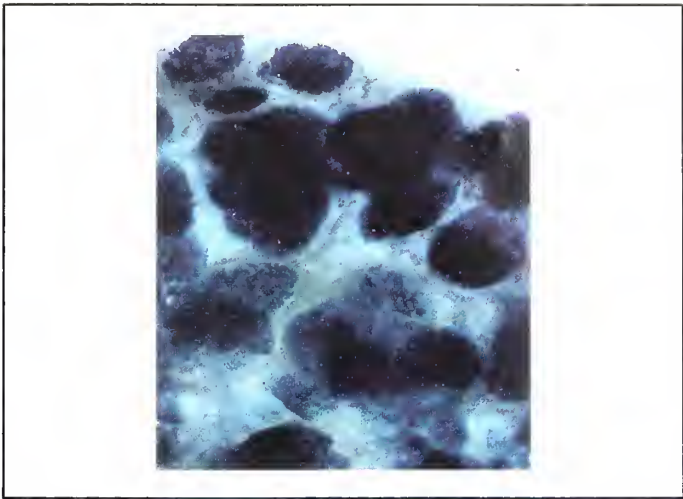


Fig. 3. Whole mount preparation of human breast tissue of a 35-year-old parous woman containing lobule 3. Toluidine blue, $\times 25$.

Table 1. Characteristics of the lobular structures of the human breast

Structure	Lobular area,* mm ²	No. of ductules per lobule†	Area of each ductule,‡ mm ²	No. of cells/ cross-section§
Lob 1	0.048 \pm 0.044	11.2 \pm 6.3	0.232 $\times 10^{-2}$	32.4 \pm 14.1
Lob 2	0.060 \pm 0.026	47.0 \pm 11.7	0.167 $\times 10^{-2}$	13.1 \pm 4.8
Lob 3	0.129 \pm 0.049	81.0 \pm 16.6	0.125 $\times 10^{-2}$	11.0 \pm 2.0

*Student's *t* tests were done for all possible comparisons. Lobular (Lob) areas showed significant differences between Lob 1 versus Lob 3 and Lob 2 versus Lob 3 ($P < .005$).

†The number of ductules per lobule was different ($P < .01$) in all the comparisons.

‡The area of each ductule per cross-section was significantly different in ductules of Lob 1 versus Lob 2 and Lob 3 ($P < .01$).

§The number of cells per cross-section was significantly different in ductules of Lob 1 versus Lob 2 and Lob 3 ($P < .01$). Modified from (63).

factors, responding selectively to given hormonal stimuli with either cell proliferation or differentiation. The type of response elicited is, in turn, modulated by specific topographic characteristics of the mammary parenchyma (70–78). In either case, the response of the mammary gland to these complex hormonal and metabolic interactions results in developmental changes that permanently modify both the architecture and the biologic characteristics of the gland (72,73). Among all of the complex hormonal influences, estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium (71,72). Estradiol acts locally on the mammary gland, stimulating DNA synthesis and promoting bud formation. Although the influence of estrogens on the proliferative activity of mammary epithelial cells has been traditionally considered to be mediated by at least three different mechanisms, a receptor-mediated (79–85), an autocrine/paracrine loop (85), and/or a negative feedback (86), it is generally accepted that the biologic activities of estrogens are mediated by the nuclear estrogen receptor (ER) that, on activation by cognate ligands, forms a homodimer with another ER-ligand complex and activates transcription of specific genes containing the estrogen response elements (87). According to this classic model, the biologic responses to estrogens are mediated by a well-characterized ER.

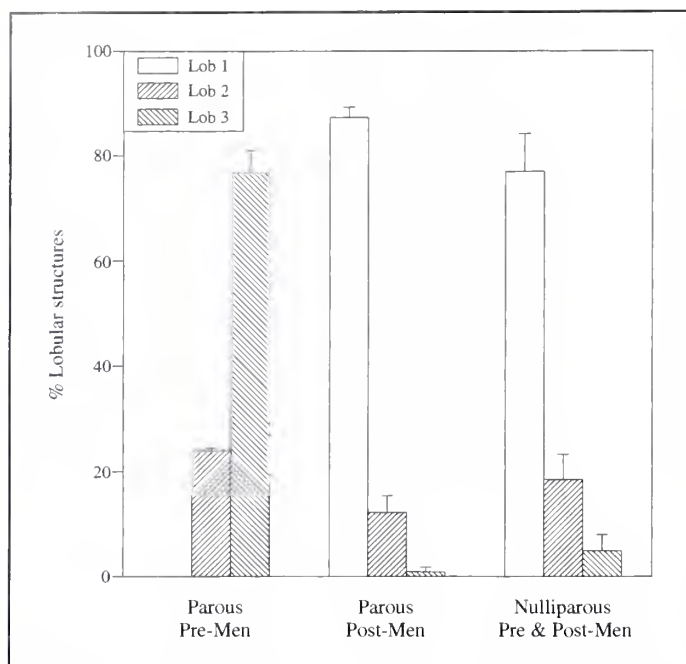


Fig. 4. Percentage of lobules (Lob) type 1 (Lob 1), type 2 (Lob 2), and type 3 (Lob 3) in the breasts of parous premenopausal (Pre-Men.), parous postmenopausal (Post-Men.), and of nulliparous premenopausal and postmenopausal (Pre & Post-Men) women.

The recent cloning of a new type of ER, the ER β from the rat (88), mouse (89), and human (90) tissues, has required researchers to rename the traditional ER as ER α . The presence of ER α in target tissue or cells is essential to their responsiveness to estrogen action. In fact, the expression levels of ER α in a particular tissue have been used as an index of the degree of estrogen responsiveness (91). A vast majority of human breast carcinomas are initially positive for ER α , and their growth can be stimulated by estrogens and inhibited by antiestrogens (92–94). ER β and ER α share high sequence homology, especially in the regions or domains responsible for specific binding to DNA and the ligands (88–90). ER β can be activated by estrogen stimulation and blocked with antiestrogens (88,90,95). On activation, ER β can form homodimers as well as heterodimers with ER α (95–99). The existence of two ER subtypes and their ability to form DNA-binding heterodimers suggests three potential pathways of estrogen signaling: via the ER α or ER β subtype in tissues exclusively expressing each subtype and via the formation of heterodimers in tissues expressing both ER α and ER β (97). In addition, estrogens and antiestrogens can induce differential activation of ER α and ER β to control transcription of genes that are under the control of an API element (100).

The importance of the integrity of ER α in the mammary gland has been clearly elucidated by using the α -ER knock-out (KO) mice (101). The mammary glands of these animals are poorly developed. Nonetheless, the α -ERKO mammary gland appears to possess the intrinsic tissue components necessary for pubertal development and pregnancy-induced maturation, but it fails to develop because of the loss of multiple stimuli that are downstream of ER α action (101). Unlike the dramatic underdevelopment observed in the mammary gland of the α -ERKO mouse, no such phenotype is observed in adult β -ERKO females (101). Although these studies are important for our understanding of the role of both ERs, extrapolation to the human breast

needs to be carefully done because these genetic-engineered mice reflect only a portion of the functional complexity of the human situation.

Progesterone is another major, although controversial, player in mammary gland biology. This ovarian steroidal hormone also acts, in conjunction with estrogen, through its specific receptor PgR in the normal epithelium for regulating breast development. The role of these hormones on the proliferative activity of the breast, which is indispensable for its normal growth and development, has been for a long time, and still is, the subject of heated controversies. Although estrogen is known to stimulate cell proliferation, the breast epithelium of sexually mature and normally cycling women does not exhibit maximal proliferation during the follicular phase of the menstrual cycle (77,78,102–106), when estrogens reach peak levels of 200–300 pg/mL and progesterone is less than 1 ng/mL (107). Instead, the breast epithelium exhibits its maximal proliferative activity during the luteal phase, when progesterone levels reach 10–20 ng/mL and estrogen levels are twofold to threefold lower than those observed during the follicular phase (107). These observations are puzzling when analyzed to the light of *in vitro* and experimental data because estrogen stimulates the proliferation of cultured breast cells and breast tissues implanted in athymic nude mice. Progesterone, however, has no effect or even inhibits cell growth in the same models (105,106).

In addition to its response to circulating hormones, the proliferative activity of the mammary epithelium in both rodents and humans varies with the degree of differentiation of the mammary parenchyma (68,71–74,108,109). In humans, the highest level of cell proliferation is observed in the undifferentiated Lob 1 present in the breast of young nulliparous females (71–74). The progressive differentiation of Lob 1 into Lob 2 and Lob 3, occurring under the hormonal influences of the menstrual cycle, and the full differentiation into lobules type 4 (Lob4) as the result of pregnancy leads to a concomitant reduction in the proliferative activity of the mammary epithelium (68,71–74,108,109).

The relationship of lobular differentiation, cell proliferation, and hormone responsiveness of the mammary epithelium is just beginning to be unraveled. Of interest, the content of ER α and PgR in the lobular structures of the breast is directly proportional to the rate of cell proliferation. These three parameters are maximal in the undifferentiated Lob 1, decreasing progressively in Lob 2, Lob 3, and Lob 4 (Fig. 5, Table 2). The determination of the rate of cell proliferation, expressed as the percentage of cells that stain positively with Ki67 antibody, has revealed that proliferating cells are predominantly found in the epithelium lining ducts and lobules and less frequently in the myoepithelium and in the intralobular and interlobular stroma. Ki67-positive cells are most frequently found in Lob 1 (Fig. 5, Table 2). The percentage of positive cells is reduced by threefold in Lob 2 and by more than 10-fold in Lob 3 (Figs. 5 and 6, Table 2) (73,110). ER α - and PgR-positive cells are found exclusively in the epithelium; the myoepithelium and the stroma are totally devoid of steroid receptor-containing cells. The highest number of cells positive for both receptors is found in Lob 1, decreasing progressively in Lob 2 and Lob 3 (Fig. 5, Table 2) (110).

To clarify the relationship between steroid receptor-positive cells and proliferating cells, we used a double-staining procedure, combining in the same tissue section anti-Ki67 and ER α , Ki67 and PgR, or ER α and PgR antibodies. Each antibody was

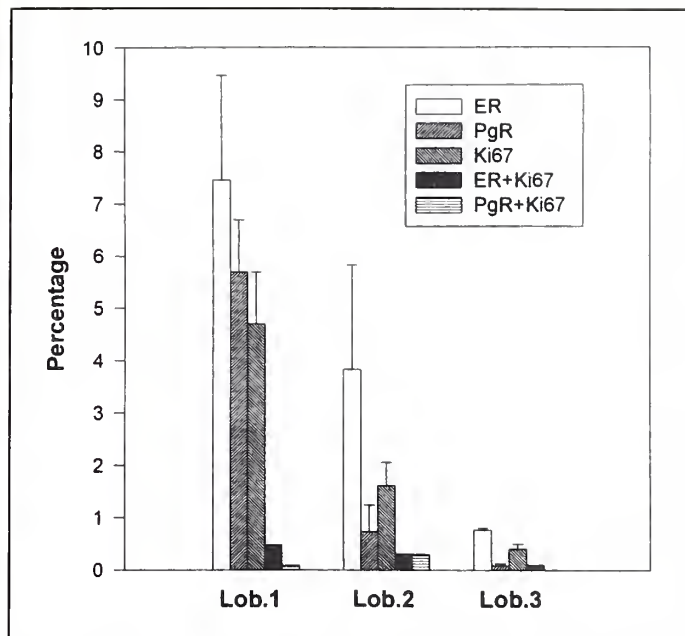


Fig. 5. Percentage of cells positive for estrogen receptor (ER), for progesterone receptor (PgR), for proliferating cells (Ki67), and for both ER and Ki67 (ER+Ki67), or PgR and Ki67 (PgR+Ki67) (ordinate). Cells were quantitated in lobule 1 (Lob 1), Lob 2, and Lob 3 of the breast (abscissa). Reproduced with permission by Kluwer Academic Publishers (110).

identified by its color reaction, brown with 3,3'-diaminobenzidine-HCl (DAB) or red with the alkaline phosphatase-vector red (110). This procedure allowed us to quantitatively determine the spatial relationship between those cells that are proliferating and those that react with either ER α or PgR antibodies. It was found that a higher percentage of cells reacted simultaneously with both ER α and PgR, appearing purple red in color (Fig. 6), whereas the number of cells positive for both ER α and Ki67 or PgR and Ki67 was very low (Table 2). The highest percentage of ER α -, PgR-, and Ki67-positive cells was observed in Lob 1 (Fig. 6, Table 2). The percentages of Ki67-, ER α -, and PgR-positive cells was reduced to 1.6%, 3.8%, and 0.7% in Lob 2, respectively. Their percentages became negligible in Lob 3 (Table 2).

Of interest was the observation that even though there were similarities in the relative percentages of Ki67-, ER α - and PgR-positive cells and in the progressive reduction in the percentage of positive cells as the lobular differentiation progressed, those cells positive for Ki67 were not the same that reacted positively for ER α or PgR (Fig. 6) (110). Very few cells, less than 0.5% in

Lob 1 and even fewer in Lob 2 and Lob 3, were positive for both Ki67 and ER α (Ki67+ER) or Ki67 and PgR (Ki67+PgR) (Table 2). Despite their low percentage, still double-labeled (Ki67+ER) cells were more numerous in Lob 1, decreasing gradually in Lob 2 and Lob 3. The percentage of cells exhibiting double labeling with Ki67 and PgR, however, were more numerous in Lob 2 than in Lob 1 but decreased to the same levels observed for ER α in Lob 3 (Table 2).

The simultaneous immunocytochemical detection of proliferating cells and of those containing ER α and PgR in normal breast tissue led us to conclude that their number varies with the degree of lobular development of the organ and that steroid receptor content is linearly related to the rate of cell proliferation. The use of a double-labeling immunocytochemical technique has allowed us to demonstrate that the expression of the receptors occurs in cells other than the proliferating cells, confirming results reported by others (104). The findings that proliferating cells are different from those that are ER α positive and PgR positive support data that indicate that estrogen controls cell proliferation by an indirect mechanism. This phenomenon has been demonstrated with the use of a supernatant of estrogen-treated ER α -positive cells that stimulates the growth of ER α -negative cell lines in culture. The same phenomenon has been shown *in vivo* in nude mice bearing ER-negative breast tumor xenografts (111,112). ER α -positive cells treated with antiestrogens secrete tumor growth factor- β that inhibits the proliferation of ER α -negative cells (113). The fact that the highest proliferative activity and the highest percentage of ER α - and PgR-positive cells are present in Lob 1 provides a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens *in vitro* (47,114), supporting as well the observations that Lob 1 is the site of origin of ductal carcinomas (115).

ARCHITECTURAL PATTERN OF THE NORMAL BREAST AT MENOPAUSE

Menopause supervenes as the consequence of the atresia of more than 99% of the 400000 follicles that are present in the ovaries of a female fetus of a gestational age of 5 months (69). Gonadotropin-releasing hormone secretion is also implicated in this phenomenon, indicating that a hypothalamic process is involved in the development of menopause. The most characteristic sign of menopause is amenorrhea, which is the result of the almost complete cessation of ovarian estrogen and progesterone production. The years leading to the final menstrual period, until menopause sets in, generally at around the age of 51 years, constitute the perimenopause. During this period, many women

Table 2. Distribution of Ki67, ER- α , and PgR-positive cells in the lobular structures of the human breast

Lobule type	No. cells	Ki67	ER	PgR	Ki67 + ER ^a	Ki67 + PgR ^a
Lob 1	19 339 ^a	4.72 \pm 1.00 ^{d,e}	7.46 \pm 2.88 ^b	5.70 \pm 1.36 ^k	0.48 \pm 0.28	0.09 \pm 0.01
Lob 2	8490 ^b	1.58 \pm 0.45 ^f	3.83 \pm 2.44 ^j	0.73 \pm 0.57 ^l	0.31 \pm 0.21	0.28 \pm 0.27
Lob 3	17 750 ^c	0.40 \pm 0.18 ^g	0.76 \pm 0.04 ⁱ	0.09 \pm 0.04 ^m	0.01 \pm 0.01	0.01 \pm 0.01

^aTotal number of cells counted in lobule 1 (Lob 1) in breast tissue samples of 12 donors; ^btotal number of cells counted in Lob 2 in breast tissue samples of 5 donors; ^ctotal number of cells counted in Lob 3 in breast tissue samples of three donors; ^dproliferative activity determined by the percentage of cells Ki67 positive, expressed as the mean \pm standard deviation. Differences were significant in ^eLob 1 versus ^fLob 2 ($t = 1.98$; $P < .05$), ^fLob 2 versus ^gLob 3 ($t = 2.27$; $P < .04$), and ^hLob 1 versus ⁱLob 3 ($t = 2.56$; $P < .01$). Estrogen receptor (ER)-positive cells were significantly different in ^jLob 1 versus ^kLob 2 and ^lLob 3 ($t = 2.04$; $P < .05$). Progesterone receptor (PgR)-positive cells were significantly different in ^mLob 1 versus ⁿLob 2 ($t = 2.27$; $P < .05$) and in ^oLob 1 versus ^pLob 3 ($t = 2.60$; $P < .03$). ^aPercentage of cells positive for both Ki67 and ER, expressed as the mean \pm SD. ^bPercentage of cells positive for both Ki67 and PgR, expressed as the mean \pm standard deviation. Reprinted with permission by Kluwer Academic Publishers (110).

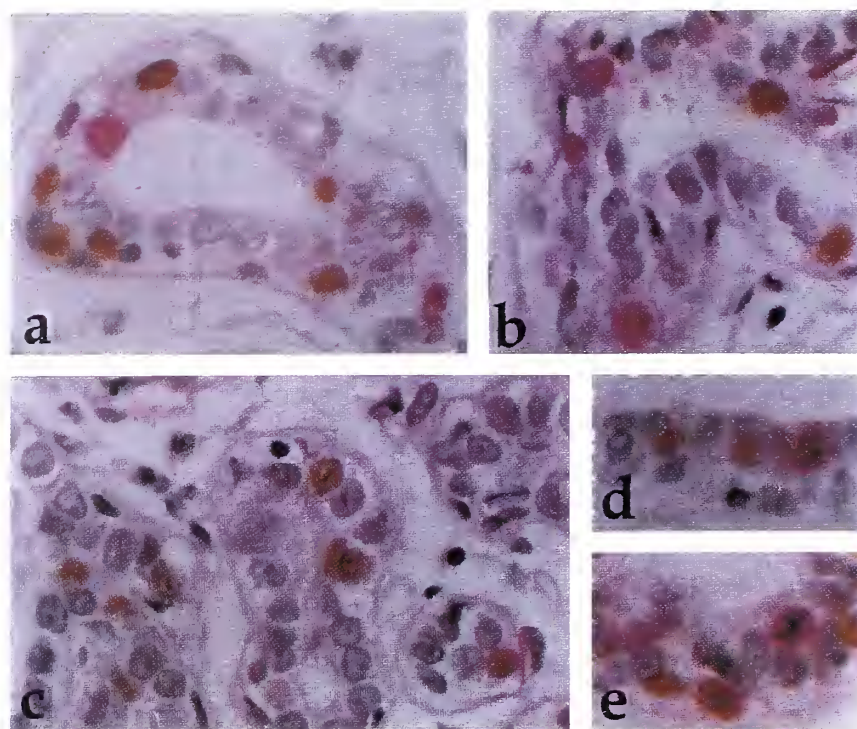


Fig. 6. Ductal epithelium of the human breast. (A) Single-layered epithelium of a lobule 1 (Lob 1) ductule contains Ki67 positive cells (brown nuclei) and estrogen receptor (ER)-positive cells (red purple nuclei; $\times 40$); (B) the single-layered epithelium lining the ductule contains brown Ki67-positive cells, and red purple PgR-positive cells. The specificity of the reaction was verified by invert-

ing the order of the stains, i.e., (C) and (D) ER-positive cells, brown, Ki67-positive cells, purple red; (E) brown nuclei of PgR-positive cells and a Ki67-positive cell in mitosis appears stained purple red (DAB-Hematoxylin; $\times 40$). Reproduced with permission by Kluwer Academic Publishers (110).

ovulate irregularly, either because the rise in estrogen during the follicular phase is insufficient to trigger a LH surge or because the remaining follicles are resistant to the ovulatory stimulus (69). The increase in human longevity occurring in our society has caused a considerable increment in the number of women that will live one third or more of their lives after menopause, a period characterized by profound ovarian hormone deprivation.

After menopause the breast undergoes regression in both nulliparous and parous women. This regression is manifested as an increase in the number of Lob 1 and a concomitant decline in the number of Lob 2 and Lob 3. At the end of the fifth decade of life, the breast of both nulliparous and parous women is composed predominantly of Lob 1 (Fig. 4) (68). These observations have led us to conclude that the understanding of breast development requires a horizontal study in which all different phases of growth are taken into consideration. For example, the analysis of breast structures at a single given point, i.e., age 50 years, would lead one to conclude that the breasts of both nulliparous and parous women are identical. However, the phenomena occurring in prior years might have imprinted permanent changes in the breast, which affect its susceptibility to carcinogenesis but are no longer morphologically observable. Thus, from a quantitative point of view, the regressive phenomenon occurring in the breast at menopause differs between nulliparous and parous women. In the breast of nulliparous women, the most predominant structure is the Lob 1, which comprises 65%–80% of the total lobule type components and their relative percentage is independent of age. Second in frequency is the Lob 2, and the least frequent structure is the Lob 3, which represent 10%–35% and 0%–5% of the total

lobular population, respectively. In the breast of premenopausal parous women, however, the predominant lobular structure is the Lob 3, which comprises 70%–90% of the total lobular component. Only after menopause the number of Lob 3 declines and the relative proportion of the three lobular types approaches that observed in nulliparous women. Full lobular differentiation only occurs in the parous women, especially in those completing full-term pregnancy at a young age, but lobular differentiation in nulliparous women seldom reaches the Lob 3 and never the Lob 4 stages (68). These differences in the pattern of breast development between nulliparous and parous women greatly explain the protective effect induced by pregnancy from breast cancer development. They also highlight the need to determine whether the undifferentiated Lob 1 of nulliparous women differ from those of the parous postmenopausal woman in their ability to metabolize estrogens or in the ability of the cells to repair genotoxic damage (116,117).

ARCHITECTURAL PATTERN OF THE BREAST WITH PROLIFERATIVE DISEASE

Our studies of the pattern of breast development in tissues devoid of mammary pathology, such as those obtained from reduction mammoplasties, led us to establish certain criteria of normality specific for a given age and parity status of the donors. In these tissues, we identified parenchymal structures exhibiting variations in the degree of differentiation, rate of cell proliferation, and content of ER α and PgR (118). To answer the question of whether breast lesions of either benign, premalignant, or ma-

lignant nature develop as a reflection of the stage of development of the breast, we compared parenchymal structures present in 33 reduction mammoplasty specimens with those found in 45 breast biopsies performed because of mammographic abnormalities or clinically suspicious breast masses (Table 3). Because the initiation of the neoplastic process is inversely related to the degree of differentiation of the breast, which in turn is a function of reproductive history, the patient populations were subdivided according to parity status (Table 3). In this study, we confirmed previous observations that in the reduction mammoplasty specimens (RM) the breast of nulliparous women of all ages was composed predominantly of Lob 1, whereas the breast of parous premenopausal women contained a higher concentration of Lob 3 (Table 3) (118). Those breast tissues obtained from biopsies had an architectural pattern different from that obtained from RM for women of comparable parity status because parous women that had a breast biopsy contained a higher percentage of Lob 1 and a lower percentage of Lob 3 than the parous population of the RM group (Table 3) (118).

The patient population that had breast biopsies was also subdivided into subgroups, based on the histopathologic diagnosis of their lesions. One group of 21 patients had no pathology present (normal breast or control group), one group of 15 patients had ductal hyperplasia (DH group), four patients had blunt duct adenosis (BDA group), and five patients had sclerosing adenosis (SAD group) (Table 4). Tissue sections from all these groups were analyzed for lobular architecture, type of pathologic lesions, and proliferative activity of the breast (Figs. 7 and 8) (118). The breast tissues of the groups classified as normal breast (control) and DH had a significantly higher percentage of Lob 1 than Lob 2 and Lob 3 ($P<.0008$ and $P<.0001$, respectively) (Fig. 7). The breast tissues containing BDA were also characterized by having a higher percentage of Lob 1, whereas the SAD group had a higher percentage of Lob 2 (Table 4, Fig. 7) (118). In all of the groups, the percentage of Lob 3 was significantly lower than that of Lob 1, although the relative percentage of Lob 3 was significantly higher in breast biopsies with BDA and SAD than in the normal breast biopsies or in those diagnosed with DH ($P<.05$) (Fig. 7) (118).

The number of proliferating epithelial cells, determined by immunostaining of the Ki67 nuclear antigen, was higher in Lob 1 than in Lob 2 and Lob 3 ($P<.001$), with a similar pattern in the normal breast, DH, and SAD groups, although the differences were not statistically significant between Lob 1 and Lob 2 in the SAD group (Fig. 8). In the BDA group, however, the rate of cell proliferation was higher in Lob 2 ($P<.01$) than in Lob 1 and Lob 3 (Fig. 8) (118). Although the proliferative activity was on an average higher in Lob 1 than in Lob 3, the differences were not

statistically significant (Fig. 8) (118). These data allowed us to conclude that breast tissues obtained from biopsies performed because of mammographic or clinical abnormalities, even in the absence of cancer, have architectural and cell kinetic patterns different from the normal breast tissues obtained from reduction mammoplasties. More important is the observation that even in those cases in which no pathology or only benign lesions were diagnosed, the pattern of breast development in biopsies was more similar to that of the cancer-bearing breast than it is to the population not requiring a biopsy. Our findings that in DH-containing biopsies Lob 1 are the most frequent structures present and have the highest rate of cell proliferation support our postulate that DH originates from Lob 1 (115). Lob 2 and Lob 3, which are the sites of origin of more differentiated lesions, such as BDA and SAD, are more prominently represented and are more proliferative in those biopsies containing these types of pathologic lesions. It is of importance to clarify that parity does not seem to influence the pattern of development in DH-containing breast tissues. A similar observation has been made in cancer-bearing breasts or in breasts of nulliparous women in terms of lobular composition.

BREAST DEVELOPMENT, HORMONES, AND THE PATHOGENESIS OF BREAST CANCER

From our studies associating normal breast development and the pathogenesis of both experimental and spontaneous mammary carcinogenesis emerged an important concept: that the Lob 1, the most undifferentiated structure found in the breast of young nulliparous women, is equivalent to the terminal ductal lobular unit, a structure originally identified by Wellings et al. (119) as the site of origin of ductal carcinomas (Fig. 9) (63,70). This observation was supported by comparative studies of normal and cancer-bearing breasts obtained at autopsy. We observed that the nontumoral parenchyma of those breasts that had developed a malignancy contain a significantly higher number of hyperplastic terminal ducts, atypical Lob 1, and ductal carcinomas *in situ* originating from Lob 1 than those breasts free of malignancies. These findings indicate that the Lob 1 is affected by both preneoplastic and neoplastic processes (115). More differentiated lobular structures have been found to be affected by neoplastic lesions as well, although they originate tumors whose histologic type and malignancy are in an inverse relationship with the degree of differentiation of the parent structure (52,115,120,121). The finding that the most undifferentiated structures originate the most aggressive neoplasms is clinically important because these structures are more numerous in the breasts of nulliparous women who are, in turn, at a higher risk of developing breast cancer (68).

Table 3. Lobular architecture of the breast—comparison of percentages of structures found in reduction mammoplasties and breast biopsies with proliferative breast disease

Group (parity)	No. of cases	Age, y	Lob 1 (%)	Lob 2 (%)	Lob 3 (%)
RM (all)	33	29.4 ± 8.2	22.45 ± 23.7 ^a	37.25 ± 28.61 ^c	38.41 ± 34.22 ^c
RM (nulliparous)	9	22.9 ± 6.7	45.87 ± 27.40	47.17 ± 22.01	6.94 ± 7.01
RM (parous)	24	31.9 ± 2.3	16.92 ± 8.26 ^e	35.45 ± 3.14 ^f	47.86 ± 33.4 ^h
PBD (all)	45	46.6 ± 1.5	65.66 ± 34.15 ^h	24.64 ± 20.64 ^d	9.68 ± 6.31 ⁱ
PBD (nulliparous)	10	42.5 ± 10.3	70.99 ± 33.3	25.26 ± 24.74	3.75 ± 1.6
PBD (parous)	35	48.9 ± 11.8	65.25 ± 37.3 ^h	21.10 ± 8.07 ^j	13.62 ± 3.10 ⁱ

The differences between ^a and ^b is $P<.0000005$; the difference between ^c and ^d is $P<.04$; between ^e and ^f is $P<.00009$; between ^g and ^h $P<1 \times 10^{-7}$; between ⁱ and ^j is $P<.07$, and between ^k and ^l is $P<.0003$. RM = reduction mammoplasty; PBD = proliferative breast disease. Reprinted with permission by JAI Press (118).

Table 4. Lobular structures found in breast biopsies

Group	Patient's age, y	No.	Lob 1 (%)	Lob 2 (%)	Lob 3 (%)
Normal control	42.28 ± 6.66	21	71.95 ± 7.09	23.02 ± 5.47	5.11 ± 3.17
Ductal hyperplasia	44.06 ± 11.37	15	87.76 ± 3.16	11.85 ± 3.10	0.24 ± 0.94
Blunt duct adenosis	37.25 ± 11.64	4	58.39 ± 19.93	31.57 ± 11.75	9.82 ± 9.82
Sclerosing adenosis	37.00 ± 3.16	5	37.92 ± 15.52	48.95 ± 15.65	11.31 ± 9.97

The ages of the patients in the four groups under study did not differ significantly. In the normal breast or control group, the percentage of lobule 1 (Lob 1) was significantly different from Lob 2 and Lob 3 ($P < .0008$); Lob 2 was significantly different from Lob 3 ($P < .07$). In the group with ductal hyperplasia, the percentage of Lob 1 was significantly different from Lob 2 and Lob 3 ($P < .000001$) and so were the differences between Lob 2 and Lob 3 ($P < .02$). The percentages of Lob 2 in the groups with sclerosing adenosis and blunt duct adenosis were significantly higher than those of the control ($P < .07$) and ductal hyperplasia ($P < .001$) groups. The percentage of Lob 3 was significantly higher in the groups with sclerosing adenosis ($P < .05$) and blunt duct adenosis ($P < .05$) than in those breast tissues containing ductal hyperplasia. Reprinted with permission by JAI Press (118).

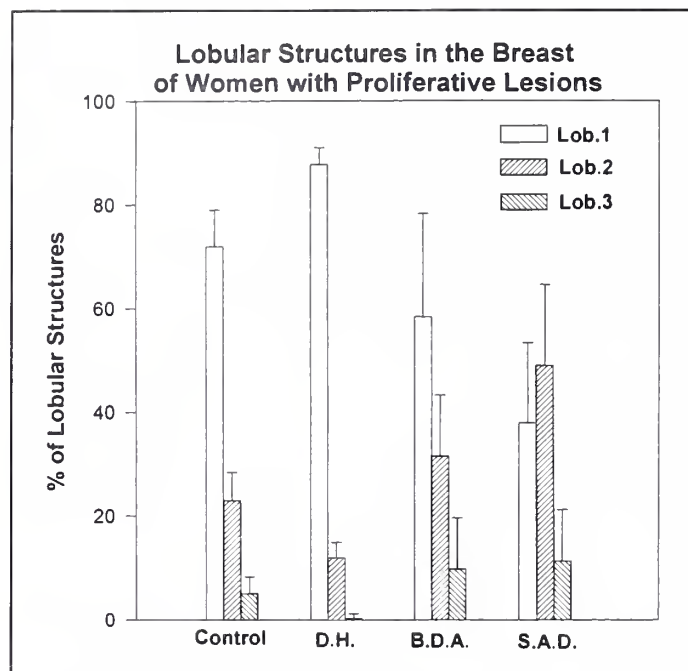


Fig. 7. Histogram showing the relative percentage of lobule 1 (Lob 1), Lob 2, and Lob 3 (ordinate) present in breast biopsies of women with proliferative lesions. D.H. = ductal hyperplasia; B.D.A. = blunt duct adenosis, and S.A.D. = sclerosing adenosis. Reproduced with permission by JAI Press (118).

The analysis of the nontumoral breast tissues from cancer-bearing lumpectomy or mastectomy specimens reveals that the breasts in nulliparous women have an architecture dominated by Lob 1, being their overall architecture similar to that of nulliparous female free of mammary pathology (120,122). Although the breast tissues of parous women from the general population contain predominantly Lob 3 and a very low percentage of Lob 1, in those parous women who have developed breast cancer, their breast tissues have also the Lob 1 as the predominant structure, appearing in this sense similar to those of nulliparous women. It is of interest that all of the parous women in our studies who had developed breast cancer had a history of late first full-term pregnancy or a family history of breast cancer. The analysis of these samples allowed us to conclude that the architecture of the breast of parous women with breast cancer differs from that of parous women without cancer. The similarities found between the architecture of the breast of nulliparous women and that of parous women with cancer support our hypothesis that the degree of breast development is of importance in the susceptibility to carcinogenesis and, furthermore, that par-

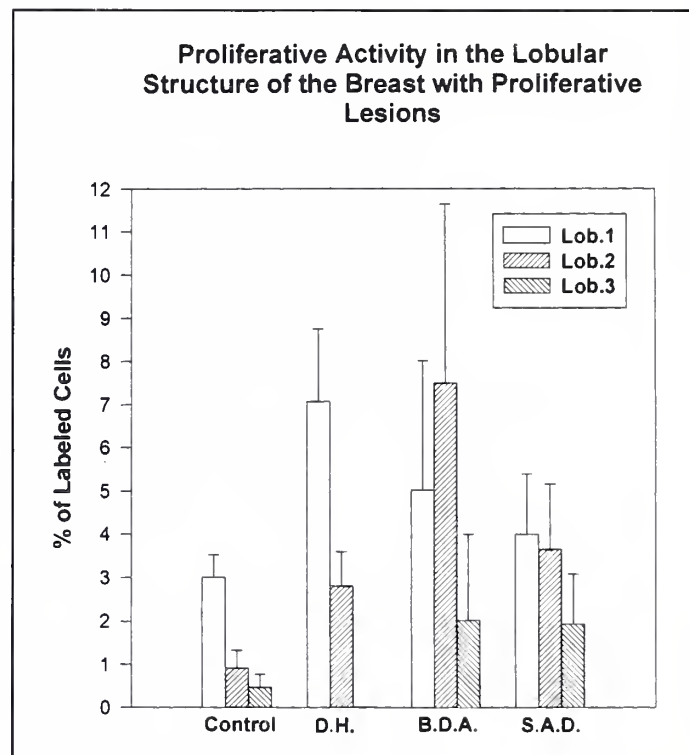


Fig. 8. Histogram showing the proliferative activity, determined as the percentage of cells reacting positively with Ki67 antibody, in the epithelium of lobule 1 (Lob 1), Lob 2, and Lob 3 (ordinate) present in breast biopsies of women with proliferative lesions. D.H. = ductal hyperplasia; B.D.A. = blunt duct adenosis, and S.A.D. = sclerosing adenosis. Reproduced with permission by JAI Press (118).

ous women who develop breast cancer might exhibit a defective response to the differentiating influence of the hormones of pregnancy (52,115,120–122).

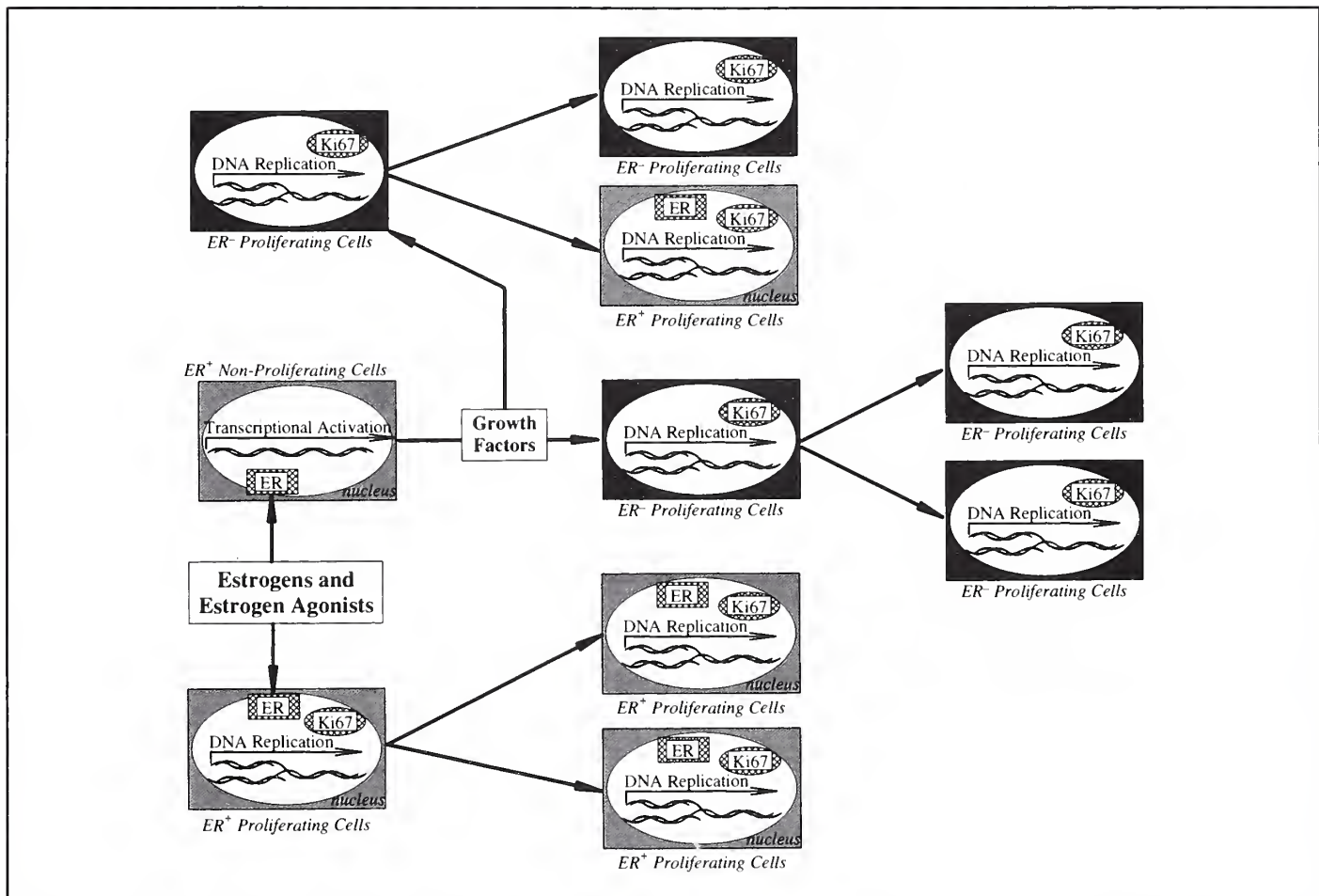
Breast cancer is a hormone-dependent malignancy. The risk of developing breast cancer has been traditionally linked to exposure to estrogen, mainly because a majority of breast cancers contain receptors for this hormone. The ER α content of a tumor is considered to be a parameter of prognostic significance (123,124). There is no information available as yet of the prognostic significance of the ER β content of a tumor. The presence of ER α -positive and ER α -negative cells and of proliferating cells, regardless of the receptor status of the normal breast, may help to elucidate the genesis of ER α -positive and ER α -negative breast cancers (125,126). It has been suggested that ER α -negative breast cancers result from either the loss of the ability

LOBULE

DUCTAL CARCINOMA IN SITU

types: ER α -positive cells that do not proliferate, ER α -negative cells that are capable of proliferating, and a small proportion of ER α -positive cells that can proliferate as well (Fig. 10) (110). Therefore, estrogen might stimulate ER α -positive cells to pro-

Therefore, estrogen might stimulate ER α -positive cells to pro-



to produce a growth factor that, in turn, stimulates neighboring ER⁻ cells capable of proliferating. ER⁺Ki67⁺ cells that can proliferate and could be stimulated by estrogen to originate ER⁺ daughter cells or probably tumors. ER⁻ cells may convert to ER⁺ cells during neoplastic transformation. Adapted from (110).

duce a growth factor that might, in turn, stimulate neighboring ER α -negative cells to proliferate (Fig. 10) (110). In the same fashion, the small proportion of cells that are ER α -positive and can proliferate could be the source of ER α -positive tumors. The possibility exists, as well, that the ER α -negative cells convert to ER α -positive cells. The newly discovered ER β opens the possibility that those cells traditionally considered to be negative for ER might be positive for ER β (88–90,128–131). It has recently been found that ER β is expressed during the immortalization and transformation of ER-negative human breast epithelial cells (Fig. 11) (131), supporting the hypothesis of a conversion from negative to positive receptor cell. The functional role of ER β -mediated estrogen signaling pathways in the pathogenesis of malignant diseases is essentially unknown. ER β -mediated mechanisms have been implicated in the increased PgR expression in the dysplastic acini of the dorsolateral rat prostate in response to treatment with testosterone and estradiol-17 β (132). ER β has been detected either alone or co-expressed with ER α in

both normal and cancerous human breast tissues and breast cell lines (129–131). These observations suggest the possibility that ER α and ER β proteins interact with each other and discriminate between target sequences leading to differential responsiveness to estrogens. In addition, estrogen responses mediated by ER α and ER β may vary with different composition of their co-activators that transmit the effect of ER-ligand complex to the transcription complex at the promoter of target genes (133).

Although the receptor-mediated mode of estrogen action is the most widely studied and accepted, evidence is mounting that a membrane receptor, coupled to alternative second messenger signaling mechanisms, is operational and may stimulate the cascade of events leading to cell proliferation (134,135). This knowledge suggests that ER α -negative cells found in the human breast may respond to estrogens through this one pathway or other known or as yet undiscovered pathways. Definitely more studies need to be done in this direction, especially when taking into consideration that, in the normal breast, the proliferating and steroid hormone receptor-positive cells are not the same. This finding has opened new possibilities for clarifying the mechanisms through which estrogens stimulate cell proliferation for initiating the cascade of events leading to cancer.

There is evidence as well that estrogen may not need to activate its nuclear receptors to initiate or promote breast carcinogenesis. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates [(18–43), Chapters 4 and 5]. The two major endogenous estrogens, estradiol-17 β (E $_2$) and estrone (E $_1$), are continuously interconverted by 17 β -hydroxylase. They are generally metabolized via two major pathways: hydroxylation at C-16 α position and at the C-2 or C-4 positions (16,20,21). The carbon position of the estrogen molecules to be hydroxylated differs among various tissues, and each reaction is probably catalyzed by various CYP isoforms (136). For example, in MCF-7 human breast cancer cells, which produce catechol estrogens (CE) in culture (23,34), CYP 1A1 catalyzes hydroxylation of E $_2$ at C-2, C-15 α , and C-16 α . CYP 1A2 predominantly at C-2 (22), and a member of the CYP 1B subfamily at C-4 (24–26). The hydroxylated estrogens are CE that have a very short half-life *in vivo* because of rapid inactivation via monomethylation at the 2-, 3- or 4-hydroxy (OH) groups catalyzed by blood-borne catechol-O-methyltransferase (COMT) [(20,27,28), Chapter 6]. Steady-state concentrations of CE are determined by the CYP-mediated hydroxylations of estrogens and COMT-catalyzed methylation of catechols (22). An increase in CE because of either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their auto-oxidation to semiquinones and subsequently quinones. These two compounds are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and, thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of CE (29). This pathway still needs to be demonstrated in normal breast epithelial cells.

Collectively, the alternative pathways described above offer new paradigms for determining the role of estrogens as endogenous carcinogens and for clarifying whether they act as initiators or promoters of the neoplastic process. Many questions remain to be answered, such as are there differences in the levels of aromatases, sulfotransferases, CYP 1A1, or CYP 1B1 present in the differentiated breast of parous women and those found in

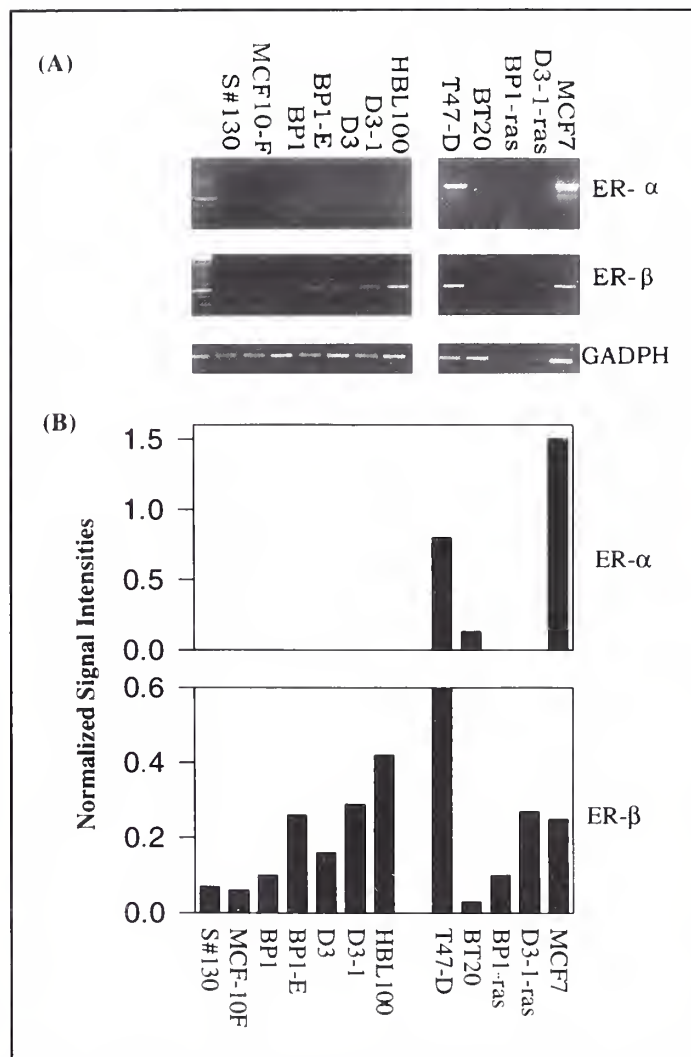


Fig. 11. Expression of ER α or ER β in mortal, immortal, chemically transformed, and neoplastic human breast epithelial cells. (A) Representative picture of the gel for ER α or ER β reactions. (B) Plots represent the average value from three independent RT-polymerase chain reaction reactions for each cell line. Signal intensities of the ER α or ER β products for each cell line were normalized using glyceraldehyde phosphate dehydrogenase (GAPDH) products to produce arbitrary units of relative abundance. Reproduced with permission by the *International Journal of Oncology* (131).

the poorly differentiated gland of young nulliparous women? By the same token, are the oxidative adduct byproducts of the metabolism of estrogens to CE_s, semiquinones, and quinones different in various types of lesions found in the breast? These are important questions that need to be answered to fully understand the role of estrogens in breast carcinogenesis.

THE *IN VITRO* MODEL OF BREAST CANCER

Culture of Human Breast Epithelial Cells *In Vitro*

Under *in vitro* conditions, HBECs have a life span comparable to that of adult human fibroblasts (30–64 doublings) when cultured in a medium supplemented with bovine pituitary extract and a standard concentration of calcium (1.05 mM) (53,54). However, when the concentration of calcium (Ca²⁺) in the medium is reduced to 0.04 mM (low Ca²⁺), the growth and population longevity of the cells are profoundly affected (56–59). HBECs cultured under low Ca²⁺ conditions divide for periods of more than 1000 days and for more than 50 generations of linear growth, without expressing terminal differentiation. Similar effects have been observed in other cell types, such as WI-38 (137), mouse epidermal (138–140), and rat esophageal epithelial cells (141). In addition, under these conditions HBECs maintain a normal diploid karyotype and human breast epithelial characteristics, including morphologic and ultrastructural features, as well as formation of domes and duct-like structures in collagen. They also express specific keratin filaments and milk fat globule membrane antigen (50,53,57). The behavior of HBECs *in vitro*, however, is in great part modulated by the biologic conditions of the breast from which they were obtained. Cells obtained from lobules varying in their degree of differentiation show variations in their *in vitro* growth properties (53,57,58,114). The number of doublings is higher in those HBECs derived from less differentiated breast tissues, i.e., the Lob 1 of young nulliparous women, which also exhibit a greater rate of cell proliferation (47,114). Furthermore, these HBECs are more susceptible to be transformed *in vitro* by etiologically important environmental chemical carcinogens, a phenomenon that does not occur in cells obtained from the more differentiated Lob 3 of older and parous women (47,114). These observations indicate that both the *in vitro* growth characteristics of HBECs and their susceptibility to be transformed by chemical carcinogens are profoundly influenced by the degree of lobular differentiation and the rate of cell proliferation of the breast epithelium *in vivo*.

Immortalization of Human Breast Epithelial Cells

As previously indicated, normal HBECs maintained *in vitro* senesce after 10–20 passages. Very few immortal breast epithelial cell lines of nonmalignant origin have been established (50,142–145). Among them, MCF-10, a HBEC line derived from a primary culture of sample S#130, designated as MCF-10M, became immortalized after culture in low Ca²⁺ medium for more than 2 years. Two immortal cell lines arose spontaneously, MCF-10A and MCF-10F (50,145). The immortalization of these cells was characterized by their continuous growth in conventional, 1.05 mM Ca²⁺ (also called high Ca²⁺ medium) without entering into senescence after more than 20 passages *in vitro*. Although the growth curves of MCF-10M, MCF-10A, and MCF-10F cells were similar when grown in low- and high-Ca²⁺ media, MCF-10M cells were unable to continue growing in high-Ca²⁺ medium after the 20th passage, whereas the immortal MCF-10A and MCF-10F cells grew indefinitely under high Ca²⁺ conditions. An important difference between MCF-10M and its immortalized derivatives was their survival efficiencies in soft agar (Table 5), even though there were no significant differences in survival efficiencies among these cell types when they were seeded in agar-methocel (Table 5). None of the three cell types formed colonies in soft agar or in agar-methocel. Instead, the neoplastic breast epithelial cell line MCF-7 had a significantly higher survival efficiency, and they formed colonies in both culture conditions (Table 5).

The immortalized cells MCF-10A and MCF-10F are bona fide HBECs, expressing epithelial sialomucins and keratins usually reported in human breast (50,145). Ultrastructurally, the cells appear low cuboidal, with their lateral borders joined by numerous desmosomes and the free surfaces are lined by short microvilli. When plated on plastic surfaces, they grow in monolayers forming domes. In a collagen matrix and under the control of hormones and growth factors the cells form ductular structures. They lack anchorage-independent growth and are not tumorigenic in severe compromised immune deficient mice. Cytogenetic analysis of MCF-10M cells showed a normal diploid karyotype, whereas the immortalized MCF-10A and MCF-10F cells had a near diploid karyotype. Genotypically, these cells were demonstrated to be of human origin by DNA hybridization with probes for highly polymorphic sequences, such as the hypervariable single-copy gene PUM. The relationship of MCF-10A and MCF-10F cells to a specific donor was demonstrated by hybridization of identical size HaeIII fragments with a M13

Table 5. Growth characteristics of HBEC grown in soft agar and in agar-methocel*

Cells	Plating efficiency (%)		Survival efficiency (%)		Colony efficiency (%)	
	SA	AM	SA	AM	SA	AM
MCF-10M	26.3 ± 8	31.1 ± 9	51.8 ± 21	93.9 ± 45	0.0	0.0
MCF-10A	41.7 ± 1	44.5 ± 14	212.1 ± 20	62.9 ± 43	0.0	0.0
MCF-10F	9.8 ± 5	21.0 ± 7	180.0 ± 96	91.9 ± 34	0.0	0.0
MCF-7	42.8 ± 6	50.7 ± 14	468.4 ± 57	672.1 ± 53	3.6 ± 0.8	6.72 ± 1.2

*The mortal human breast epithelial cells (HBECs) MCF-10M, immortal MCF-10A and MCF-10F, and malignant MCF-7 cells (1 × 10⁴/well) in 0.28% agar (soft agar; SA) or 0.8% methylcellulose (agar-methocel; AM) were seeded in 24-well plates in which the flat polystyrene had been precoated with a solidified layer of 0.9% agar in culture medium without supplements. The plates were incubated at 37°C and the medium replenished twice a week. Twenty-four hours after planting, all cultures were examined for cell aggregates that might bias the final results. Twenty-one days after planting, viable cells and colonies ranging in size from 50 to 250 μm in diameter, as determined with a micrometer disc, were counted under an inverted phase contrast microscope. The results were expressed as plating efficiency (i.e., number of cells 24 hours after plating per 100 cells plated), survival efficiency (i.e., number of cells survived 21 days after plating per 100 cells plated), and colony efficiency (i.e., number of colonies formed 21 days after plating per 100 cells plated).

probe that detects multiple hypervariable minisatellites (145). The immortal MCF-10 cells do not have amplification of c-erbB2/HER-2-neu, erbA-1, int-2, int-1 or mutated c-Ha-ras-1 gene and do not contain SV40 antigen. These characteristics make this cell line the most near to a normal HBEC line available. Undoubtedly, the establishment of the immortal cell line MCF-10F that arose spontaneously from the mortal diploid HBECs without viral or chemical intervention provides an important tool for understanding how chemical carcinogens are able to induce transformation phenotypes. At the same time, it also provides the basis for understanding the differences in the processes that control senescence, immortalization, and malignancy.

Neoplastic Transformation of Human Breast Epithelial Cells With Chemical Carcinogens

Exposure of primary cultures of HBECs to chemicals such as benzo[a]pyrene (BP), *N*-methyl-*N*-nitrosourea (NMU), and 7,12-dimethylbenz[a]anthracene (DMBA), all known to be carcinogenic in animals (115), reveals that their susceptibility to be transformed is modulated by host factors and by specific characteristics of the cells. Treatment of MCF-10F cells with BP and DMBA has given rise to a series of chemical-carcinogen-initiated cell lines, each one expressing a well-defined transformed phenotype. The transformed cells express anchorage independence, loss of ductule-like formation in collagen gel, and increase in chemotactic and invasive properties, tumorigenicity in heterologous hosts (e.g., BP1E tumor cell lines) (47,49,146). Thus, the highest susceptibility of HBECs to be transformed has been found to be associated with a high rate of cell proliferation and undifferentiated condition of the donor organ *in vivo* (114), immortalization prior to exposure to the carcinogens (47), and inherited predisposition to breast cancer, as revealed by our studies of HBECs obtained from prophylactic mastectomies performed in women with familial history of breast cancer and genetic predisposition, as evidenced by linkage analysis (147).

Primary cultures established from outgrowths of organoids obtained from prophylactic mastectomies received two 24-hour treatments with BP or DMBA in a week. After 30 days, the cells were plated in agar-methocel for evaluation of anchorage-independent growth. By day 21 postplating, viable cells and colonies, ranging in size from 50 to 250 mm in diameter, were counted. Results were expressed as survival efficiency, colony efficiency, and colony size. Survival efficiency (Fig. 12, A) and colony efficiency (Fig. 12, B) in agar-methocel were calculated as number of cells that survived per 100 cells plated and as number of colonies formed per 100 cells plated, respectively. The average size of the colonies was measured with a micrometer disc under an inverted phase contrast microscope (Fig. 13).

BP and DMBA did not affect survival efficiency but significantly increased colony efficiency of treated cells. The colonies formed by treated cells showed considerable anchorage-independent growth during the 21-day assay period (Fig. 12, A and B) (147). Because the formation of colonies in agar is generally construed as an indication of anchorage-independent growth, a hallmark of neoplastic cells, our results clearly indicated that HBECs from women with familial history of breast cancer manifested phenotypic changes indicative of initial stages of neoplastic transformation in response to the treatment with carcinogens (147). In contrast, the same treatments when applied to HBECs of women without familial history of breast cancer

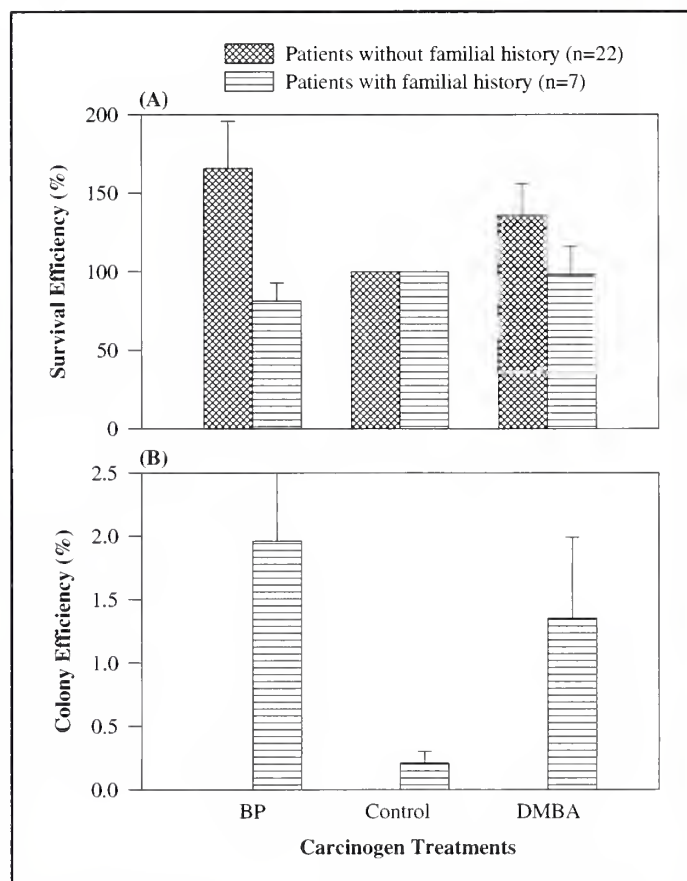


Fig. 12. Survival efficiency (A) and colony efficiency (B) in agar-methocel of chemical carcinogen-treated human breast epithelial cells (HBECs) from women with or without familial history of breast cancer (mean \pm SD). Survival efficiencies were calculated as a percentage of the values of controls for each sample to minimize derivations in responses among samples and to compare with the values of two previous studies on carcinogen-treated HBECs from women with no familial history of breast cancer. Reproduced with permission by the Society for *In Vitro* Biology (147).

induced phenotypic alterations indicative of partial transformation only, such as increased survival efficiency in agar methocel (Fig. 12, A and B), which is perceived to precede the acquisition of anchorage independence.

Our observations led us to conclude that genetic predisposition in women with familial history of breast cancer confers inherited susceptibility to environmental chemical carcinogens (147). However, induction of the full spectrum of transformed phenotypes by chemical carcinogens requires immortalization of the cells prior to exposure to the carcinogens. It is not known how estrogens or other endocrine disrupters influence the susceptibility of the epithelial cells to undergo neoplastic transformation, or whether these cells will express transformation phenotypes when estrogen is metabolized or interacts with specific nuclear receptors.

Molecular Basis of Cell Immortalization and Neoplastic Transformation

Genomic Alterations

Genomic analysis of MCF-10F cells, using single-strand conformational polymorphism technique revealed the presence of a variant band in one of the heterozygous alleles of *TP53*. On

DNA sequencing, a point mutation (TAG \rightarrow TTA) of codon 254 in exon 7 of this gene was detected (Table 6) (148). There was a coexistence of a mutation of *TP53* and instability of microsatellite DNA in the intragenic *TP53* in these cells, manifested by additional bands with slower mobility. We used polymerase chain reaction (PCR) amplification of microsatellite DNA length polymorphism to detect allelic loss as well as microsatellite instability (MSI). These microsatellites are highly polymorphic, flanked by unique sequences that can serve as primers for PCR amplification. They have been proven to be useful markers for investigating multiple areas of MSI and loss of heterozygosity (LOH) and should be applicable to allelotyping as well as regional mapping of deletions in specific chromosomal regions. We have studied 466 markers that represent approximately 4.6% of the 10 000 microsatellite markers identified. Microsatellite PCR analysis of MCF-10F cells did not reveal LOH with any of the markers analyzed in this study when compared with their parental MCF-10M cells. However, MCF-10F cells did show MSI in chromosome 11 in the locus D11S392 and in chromosome 17 in the loci represented by markers D17S849, *TP53*, D17S786, and D17S520 (149).

Neoplastic transformation of MCF-10F cells with chemical carcinogens (e.g., BP1 and BP1E cells) is associated with genetic instability on chromosomes 11 and 13, in addition to that observed on chromosome 17, which has been detected in association with immortalization (150–153). MSI was found on chromosome 11 by using marker D11S912 and expressed as an allelic expansion in the BP1 and BP1E cells, representing an additional location affected in the early stage of transformation of HBECs (Fig. 14) (151). On chromosome 13, MSI was found in both BP1 and BP1E cells by using markers D13S260 and D13S289 at 13q12–13 (flanking the *BRCA2* locus) (Fig. 14) (150). In addition, we have also observed other genomic alterations on chromosomes 9 and 16 (154).

Activation of Telomerase in the Immortalized MCF-10F Cells

There is evidence that the repetitive TTAGGG sequences located at the ends of human chromosomes (i.e., telomeres) may act as a molecular mitotic clock (155). It is generally believed that each successive genomic replication is accompanied by gradual shortening of 50–200 base pairs (bp) because of incomplete replication of the 3' ends, and cellular senescence occurs

when telomeres reach a critically short length that replication of the genome cannot be maintained (156). The stabilization of the telomeric sequences at the ends of chromosomes, which is required for the continuous proliferation of immortal cells, involves the activation of the enzyme telomerase, which adds TTAGGG repeats to the 3' ends of chromosomes (157,158). The genetic nature of cellular senescence implicates activation of telomerase as a key element of cell immortalization (144,158). Elevated levels of telomerase activities have been detected in a number of immortal cell lines and human tumor tissues (159,160). We have observed telomerase activity in immortal MCF-10F but not in the mortal MCF-10M cells (161), suggesting that telomerase activation may play a role in the spontaneous immortalization of MCF-10F cells.

Increase of H-Ferritin in MCF-10F Cells

In efforts to identify genes underlying the process of immortalization, we have performed subtractive hybridization and differential display analysis between immortal MCF-10F and its parental mortal MCF-10M cells. With the use of a 10F⁽⁺⁾/10M⁽⁻⁾ subtractive complementary DNA (cDNA) library, we isolated more than 15 clones, one of which contains sequences identical to H-ferritin (162). We observed marked increases in messenger RNA (mRNA) levels of ferritin H in immortal MCF-10F cell lines (particularly in late passages) (Fig. 15) and in tissues exhibiting an increase in growth rate, such as ductal hyperplasia, carcinoma *in situ*, and invasive carcinoma (Fig. 16) (162). An increase in transcript signal was also confirmed by *in situ* hybridization of breast tissues containing lesions representative of progressive stages of neoplastic evolution (Fig. 17). The levels of expression were undetectable in normal tissues; they increased progressively from moderately elevated in DH, a stage of cell progression from normal to neoplasia that may be a histopathologic parallel of cell immortalization, greater expression in carcinoma *in situ*, with the highest transcript levels being detected in infiltrating ductal carcinoma (Fig. 17).

Ferritin is a large protein found in most cell types of vertebrates, as well as of invertebrates, plants, and bacteria (163). The main function of ferritin is iron storage. This function, in turn, can be subdivided into iron storage for other cells (specialized-cell ferritin), iron storage for intracellular needs (normal house-keeping ferritin), and iron storage for intracellular protection

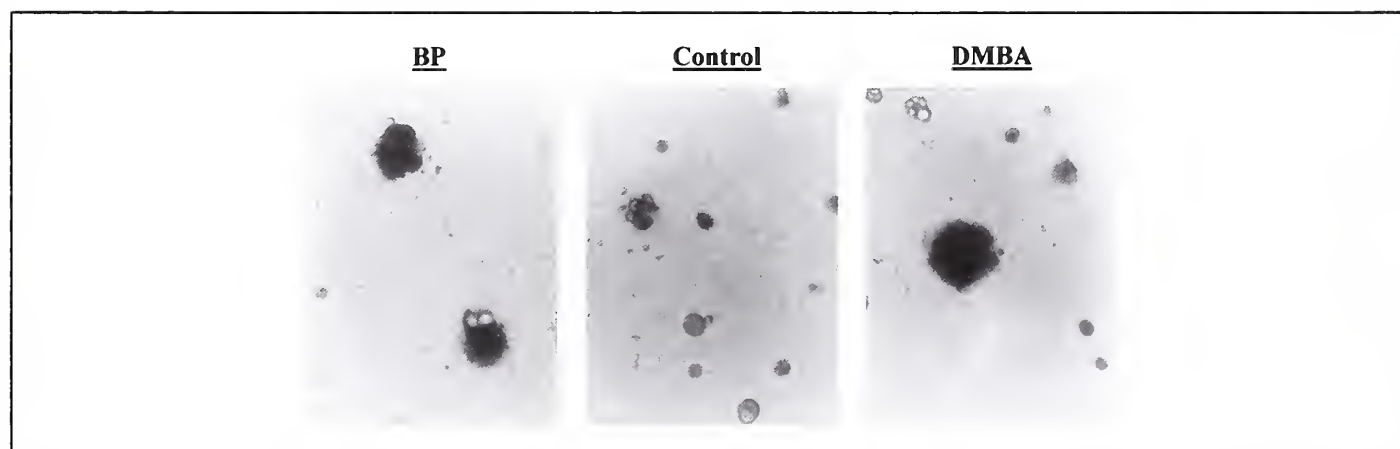


Fig. 13. Representative colonies formed in agar-methocel by chemically transformed primary cultures of human breast epithelial cells obtained from women with familial history of breast cancer. The cells were treated with the chemical carcinogens benz[a]pyrene (BP) or 7,12-dimethylbenz[a]anthracene (DMBA) prior to plating (phase contrast photograph, $\times 508$). Reproduced with permission by the Society for *In Vitro* Biology (147).

Table 6. Comparison of the sequence of p53 exon 7 in MCF-10M and MCF-10F cells

Cells	Codon No.										
	253	254	255	256	257	258	259	260	261	262	263
MCF-10M*											
Antisense	TGG	TAG	TAG	TGT	GAC	CTT	CTG	AGG	TCC	agt	cct
Sense	ACC	ATG	ATG	ACA	CTG	GAA	GAC	TCC	AGG	tca	gga
Aminoacid	Thr	Ileu	Ileu	Thr	Leu	Glu	Asp	Ser	Ser		
MCF-10F†											
Antisense	TGG	TTA	GTA	GTG	TGA	CCT	TCT	GAG	GTC	CAG	TCC
Sense	ACC	AAT	CAT	CAC	ACT	GGA	AGA	CTC	CAG	GTC	AGG
Aminoacid	Thr	Asn	His	His	Thr	Gly	Arg	Leu	Gln	Val	Arg

*MCF-10M cells exhibit a wild-type sequence.

†MCF-10F cells show a frame-shift mutation by insertion of a base at codon 254. Reprinted with permission from *International Journal of Oncology* (148).

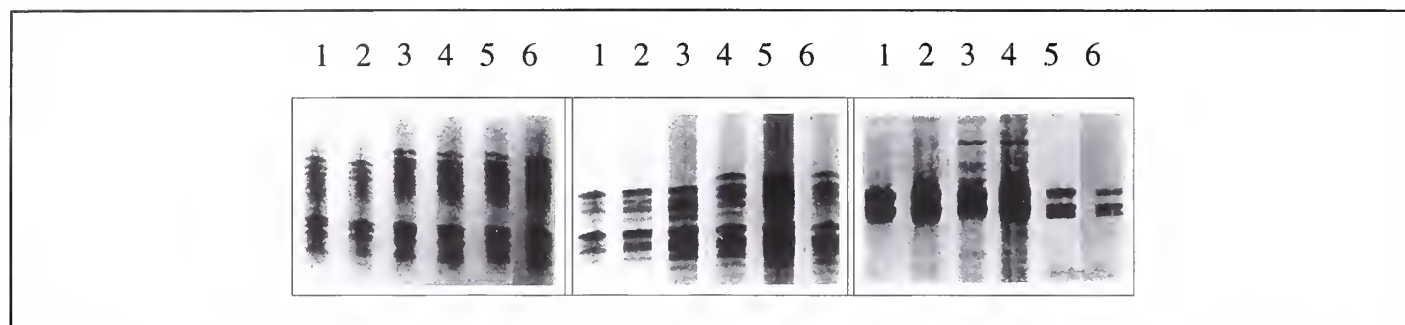


Fig. 14. Microsatellite instability detected in chromosome 11 and 13 with markers D11S9129 (left panel), D13S260 (central panel), and D13S289 (right panel) in MCF-10M (p22), lane 1; MCF-10F (P130), lane 2; BP1 (p27, p52), lane 3; BP1E (p25), lane 4; BP1E (p30), lane 5, and BP1E (P60), lane 6.

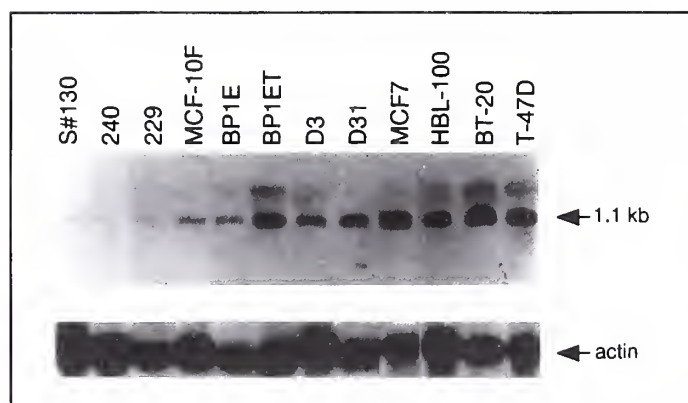


Fig. 15. Northern analysis of primary cultures of breast epithelial cells S#130, 240, and 229; immortal MCF-10F cells; benz[a]pyrene-transformed cells BP1E and BP1ET; dimethylbenz[a]anthracene-transformed cells D3 and D3-1; and the neoplastic cell lines MCF-7, HBL-100, BT-20, and T-47D. An increased signal for H-ferritin messenger RNA is observed in MCF-10F cells; a much higher intensity in BP1E, BP1ET, D3, and D3-1; and the highest signal intensity is observed in the malignant cell lines MCF-7, HBL-100, BT-20, and T-47D. Reproduced with permission by Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (162).

from iron overload (stress housekeeping ferritin) (163). Iron is required for DNA synthesis necessary for cell growth and multiplication (164–166). It is also required for electron transport and for oxygen metabolism, generating harmful activated oxygen species capable of damaging DNA, lipids, and proteins (167). The iron-catalyzed conversion of H_2O_2 is a major route to the synthesis of highly reactive OH radicals that inflict damage on the nucleotide bases of DNA, inducing mutations and increasing the risk of cancer (42,168).

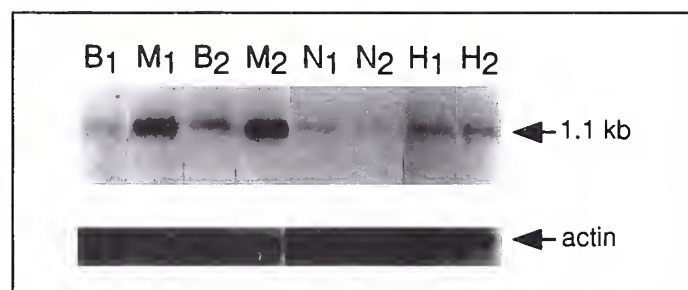


Fig. 16. Northern analysis of total RNA from breast cancer tissue (M1 and M2), normal tissue from the same patient (B1 and B2), normal control breast tissue (N1 and N2), and tissue with ductal hyperplasia (H1 and H2). A high signal intensity is seen in the malignant tissue, very low levels are detected in normal tissue samples, and an increase in signal above normal levels is seen in tissue showing ductal hyperplasia. Reproduced with permission by Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (162).

Normally, the concentration of iron capable of catalyzing these reactions is tightly controlled and regulated, and a critical homeostatic balance is achieved by the synthesis of ferritin (163,169). Many compounds, such as flavins and xanthine oxidase, are capable of reductively releasing iron from ferritin (170,171). Once released from ferritin, iron in the ferrous (Fe^{++}) state is capable of participating in free-radical reactions (Fenton reactions) leading to oxidative damage (Fig. 18). Thus, a disruption in normal iron homeostasis may lead to an increase in the level of reactive iron and to a corresponding increase in oxygen free-radical generation and DNA damage (172). Oxidative stress has also been implicated in metastasis, because it results in loss of cell adhesion, a prerequisite for cell detachment and subsequent host tissue invasion (173,174).

Progression of invasive breast cancer to the metastatic state is

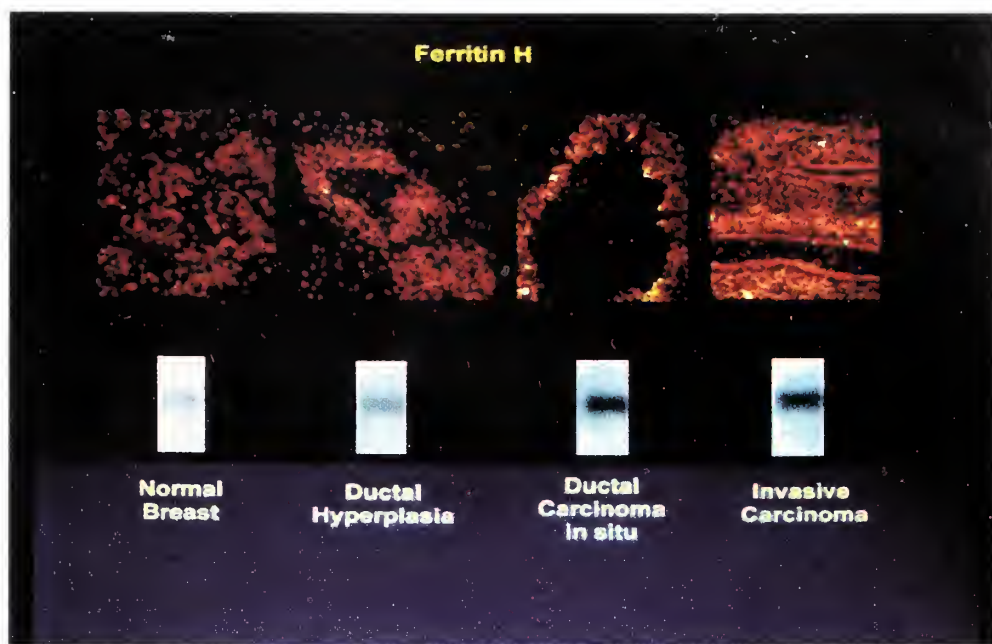


Fig. 17. *In situ* hybridization (upper row) and Northern blot analysis of Ferritin H chain messenger RNA (lower row) in normal breast lobules (Normal Breast), Ductal Hyperplasia, Ductal Carcinoma *in situ*, and Invasive Carcinoma. Tissue sections were counterstained with hematoxylin and photographed in dark field (final magnification $\times 100$).

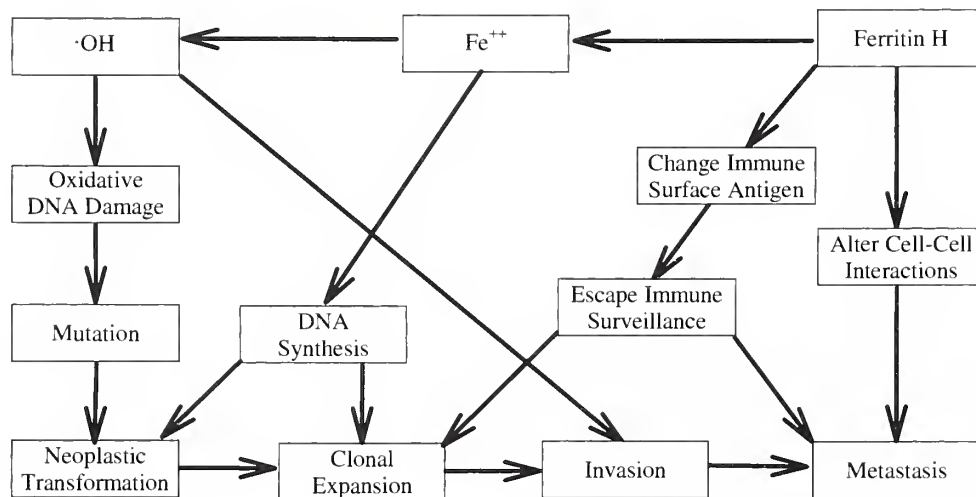


Fig. 18. The possible role of increased ferritin H chain gene in the neoplastic transformation of human breast epithelial cells. Adapted from (162).

linked to OH radical-induced DNA damage (175–177). Thus, ferritin-dependent oxidative damage to DNA may be one of the mechanisms contributing to immortalization of HBECS. An increase in ferritin H chain levels may provide iron necessary for the clonal selection and uncontrolled growth of cells. It has been shown that iron and its binding proteins participate in a variety of reactions required for cell proliferation (56,178) and are critical for the activity of the enzyme ribonucleotide reductase, a rate-limiting enzyme in DNA synthesis (165,179). Ferritin has been shown to have an immunosuppressive effect on host immune response in cancer patients (180,181). Placental isoform, an acidic form of ferritin, and its p43 super heavy chain have

been reported to be synthesized by breast cancer cells but are absent in normal breast epithelium (182). Breast cancer-associated p43 induces alterations in the expression of cell-surface molecules in neoplastic cells, which in turn could have an effect on the modulation of the cells' adhesive interactions (183). Cytokines, such as tumor necrosis factor, interleukin-1 α , and the NF- κ B family of transcription factors, specifically induce synthesis of ferritin H by selectively increasing ferritin H transcription (Fig. 18) (183–185).

Our observations that the immortalization of HBECS was associated with an increased ferritin H chain gene transcription led us to postulate that this increase might have contributed to

the immortalization of HBEC; probably through one or more of the following mechanisms: a) providing a source of iron required by rapidly dividing cells for clonal expansion, b) providing iron capable of participating in free-radical reactions leading to oxidative DNA damage and mutation, or c) affecting immune surface antigens and thus providing immortal cells a growth advantage by allowing them to escape immune surveillance (Fig. 18). However, the possibility that ferritin H chain gene induction may be a consequence of the immortalized condition of the cells, rather than its cause, cannot be ruled out. In either instance, it may prove to be a valuable marker of cell immortalization or an early indicator of malignant transformation.

Reversion of Immortalized and Transformed Phenotypes

To investigate the functional role of genomic changes on chromosomes 11 and 17 that were detected in the immortalized and transformed cells, single normal human fibroblast A9-derived chromosome 11 or 17, tagged with a neomycin-resistant gene, was transferred into 6×10^6 transformed BP1E cells. Surviving cells or clones of microcell hybrids from chromosome 11 or 17 were designated BP1E-11neo or BP1E-17neo cells. A total of 16 colonies was isolated from BP1E-11neo and BP1E-17neo cells each. The transfer efficiency in BP1E cells was approximately 2.6×10^{-6} cells (149). During a selection period of up to 6 months, BP1E-17neo cells, and to a lesser degree BP1E-11neo cells, exhibited altered cellular morphology and growth pattern, such as contact inhibition and cellular senescence (149). In addition to the acquired ability to survive in the G-418 selection medium that indicates the active function of the neomycin-resistance gene tagged on the donor chromosome 11 or 17, the physical presence of these chromosomes was further confirmed by dual-color fluorescence *in situ* hybridization (FISH) analysis. As shown in Fig. 19, A and B, the metaphases of the BP1E-11neo#145 cells were confirmed to contain an extra chromosome 11 that had a stronger signal with the painting probe (red). Anchorage-independent growth in agar-methocel gel was reduced from 17% in control BP1E cells to 7% in the BP1E-11neo#145 cells, whereas BP1E-17neo D100 cells failed

to form any colony (100% reduction), reflecting a more potent suppression of the BP1E cells by chromosome 17 than that by chromosome 11. These data indicated that the introduced chromosome 11 caused a partial growth inhibition, whereas chromosome 17 produced a nearly complete growth suppression of the BP1E cells (149). Another phenotypic reversion induced by the chromosome transfer was the recovery of the ability of cells to form ductule-like structures in collagen gel, a property exhibited by BP1E-17neo D100 cells, similar to that of MCF-10F cells, whereas BP1E cells grew in loosely arranged clusters or as isolated cells. These observations confirmed the reversion of the transformed phenotype by chromosome 17 transfer.

Microsatellite analysis showed that the preexisting instability in the parental BP1E cells at loci D17S849 (17p13.3), TP53 (17p13.1), D17S786 (17p13.1), and D17S520 (17p12.0) was reverted in BP1E-17neo D100 cells, which acquired an allelic pattern similar to that of the mortal MCF-10M cells. In contrast, the instability of these markers was not restored in the BP1E-11neo #145 cells. These data indicated a specific effect associated with transfer of chromosome 17. Surprisingly, none of the corresponding donor alleles observed in the A9-17neo cells could be detected in the BP1E-17neo D100 cells, suggesting that other untested regions of the donor chromosome 17 might be the responsible ones for the phenotypic reversion and the restored microsatellite stability.

In summary, our data provide supportive evidence for the hypothesis that MSI within or near genes can confer instability to these genes and alter their expression or functions. However, further investigation is required for determining what is their functional role in the initiation and progression of neoplasia. TP53 has been considered as the guardian of the genome by allowing cells to undergo DNA repair prior to entering a new cell cycle (186–188). The observation that introduction of an unaffected chromosome 17 can correct instability on the corresponding chromosome, including that of marker TP53, in addition to the reversion of transformed phenotypes in the transformed BP1E cells, suggests that other important genes on chromosome 17 may control this process.

FUTURE PERSPECTIVES

In the paradigm described above, it is clear that if estrogens play a role in the early stages of cell immortalization and transformation, this experimental system will allow us to demonstrate such phenomena. The demonstration of the ability of the mammary epithelial cells to metabolize estradiol and/or to accumulate “genotoxic” metabolites could profoundly influence our understanding of the neoplastic transformation of the mammary epithelium (189). Metabolic biotransformation of estradiol occurs in human mammary explant cultures composed of a mixture of epithelial and stromal cells (190,191). Treatment of normal mouse mammary epithelial cells with the mutagenic polycyclic hydrocarbon DMBA results in production of 16 α -hydroxyestrone. This predominant metabolite of estrogen increases unscheduled DNA synthesis, cellular proliferation, and anchorage-independent growth, all phenomena indicative of preneoplastic transformation (192). Because normal HBECs are susceptible to be transformed by environmental carcinogens that require metabolic activation (116,117) and many of the enzymes (e.g., CYP 1A1) that catalyze the oxidation of drugs, alkaloids, and environmental pollutants also catalyze the hydroxylation of estrogens (136,193,194), we hypothesize that HBECs, regardless

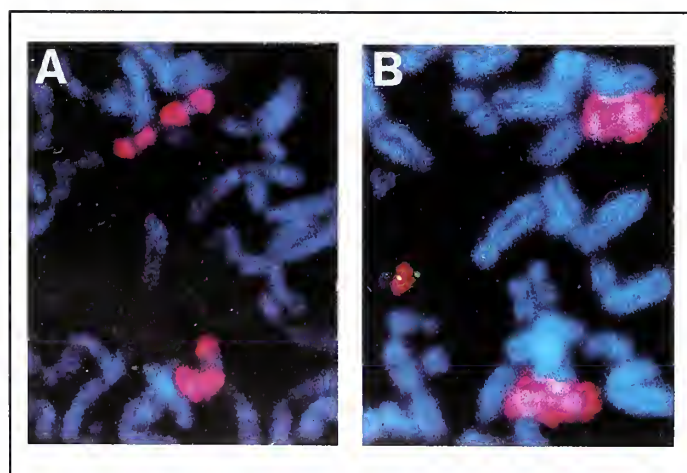


Fig. 19. Dual-color fluorescence *in situ* hybridization (FISH) for the detection of chromosome 11 in metaphases of BP1E-11neo #145 cells. (A) A representative field of a metaphase spread showing a pair of host chromosome 11 (red stained) and a donor (partial) chromosome 11 (red stained with green signal). This clone contained one extra donor chromosome 11, in addition to the pair of host chromosome 11. (B) Representative field of a metaphase spread showing a pair of chromosome 11 (red stained) in BP1E cells.

of their ER status, are capable of metabolic activation of estrogen and, thus, susceptible to estrogen-induced carcinogenesis. It is possible that the rates of metabolic activation of estrogen might vary among HBECs with different carcinogenic susceptibility. We postulate that the susceptibility of cells to be transformed by estrogens would depend on their rate of proliferation, genetic predisposition, and mortal status of the cells, rather than their ER contents, similar to what has been observed with chemical carcinogens (47,114,146,147). If these assumptions are true, the efficiency and extent of estrogen-induced neoplastic transformation will be high in immortalized MCF-10F cells, moderate in those HBECs derived from breasts of women with family history of breast cancer, and low in cells derived from the breast of parous women and of those women with no family history of breast cancer. The independence of ER contents in estrogen-induced carcinogenesis would support the postulate that metabolic activation of estrogen is involved in the neoplastic transformation of susceptible HBECs. Alternatively, estrogen or its metabolites may not initiate neoplastic transformation, but they may act by promoting neoplastic progression in chemically transformed HBECs by increasing the genomic instability of the cells. In essence, estrogen, on metabolic activation, might serve as an initiator and/or as a promoter of carcinogenesis in the human breast. However, the metabolism of estrogen in normal HBECs and the carcinogenic potential of estrogen and its metabolites in the human breast are virtually unknown.

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Chapter 2: The Role of Steroid Hormones in Prostate Carcinogenesis

Maarten C. Bosland

Carcinoma of the prostate is the most frequently diagnosed malignancy and the second leading cause of death as a result of cancer in men in the United States and in many other Western countries. Notwithstanding the importance of this malignancy, little is understood about its causes. The epidemiology of prostate cancer strongly suggests that environmental factors, particularly diet and nutrition, are major determinants of risk for this disease, and evidence is mounting that there are important genetic risk factors for prostate cancer. Human prostate carcinomas are often androgen sensitive and react to hormonal therapy by temporary remission, followed by relapse to an androgen-insensitive state. These well-established features of prostate cancer strongly suggest that steroid hormones, particularly androgens, play a major role in human prostatic carcinogenesis, but the precise mechanisms by which androgens affect this process are unknown. In addition, the possible involvement of estrogenic hormones is not entirely clear. The purpose of this overview is to summarize the literature about steroid hormonal factors, androgens and estrogens, and prostate carcinogenesis. From these literature observations, a multifactorial general hypothesis of prostate carcinogenesis emerges with androgens as strong tumor promoters acting via androgen receptor-mediated mechanisms to enhance the carcinogenic activity of strong endogenous genotoxic carcinogens, such as reactive estrogen metabolites and estrogen- and prostatitis-generated reactive oxygen species and possible weak environmental carcinogens of unknown nature. In this hypothesis, all of these processes are modulated by a variety of environmental factors such as diet and by genetic determinants such as hereditary susceptibility and polymorphic genes that encode for steroid hormone receptors and enzymes involved in the metabolism and action of steroid hormones. [J Natl Cancer Inst Monogr 2000;27:39–66]

Carcinoma of the prostate is the most frequently diagnosed malignancy and the second leading cause of death as a result of cancer in men in the United States and in many Western countries (not counting nonmelanoma skin cancer) (1). Notwithstanding the importance of this malignancy, little is understood about its causes. Steroid hormones, particularly androgens, are suspected to play a major role in human prostate carcinogenesis, but the precise mechanisms by which androgens affect this process and the possible involvement of estrogenic hormones are not clear. A causal relation between androgens and prostate cancer development is generally considered to be biologically very plausible because the vast majority of human prostate cancers are androgen sensitive and respond to hormonal therapy by temporary remission, later followed by relapse to a hormone-refractory state. The purpose of this overview is to summarize the literature about steroid hormonal factors and prostate carcinogenesis. Although the objective of this overview is not to be

comprehensive, an attempt is made to be complete, especially where crucial aspects of this hormonal involvement are concerned.

In contrast, the prostate is a rare site of tumor development in carcinogenesis bioassays in rodents (2,3) and in aging male laboratory rodents, with the exception of ventral prostatic neoplasms in some rat strains (4–9). Prostate cancer is also rare in male farm and companion animals, with the notable exception of the dog, which is the only species besides man that develops this malignancy. As will be discussed in this overview, steroid hormones can induce and can substantially enhance prostate carcinoma development in rodents, and this phenomenon has been exploited to further our knowledge about the involvement of hormonal factors and mechanisms in prostate cancer etiology.

In this overview, the epidemiologic evidence for a role of steroid hormonal factors in prostate carcinogenesis is summarized first, followed by review of experimental data, a discussion of the possible mechanisms whereby steroid hormones, androgens as well as estrogens, may be involved in prostate cancer causation, and overall conclusions and suggestions for future research. As will be demonstrated, there is no lack of hypotheses about the role of steroid hormones in prostate cancer etiology, but the available data are often contradictory and incomplete, and an in-depth overall mechanistic understanding of how steroid hormonal factors are involved in prostate carcinogenesis is very limited.

EPIDEMIOLOGIC EVIDENCE FOR INVOLVEMENT OF STEROID HORMONES

The epidemiology of prostatic cancer has been reviewed in depth elsewhere (10–15). Prostate cancer risk factors that are associated with hormonal factors are summarized in subsequent sections, and epidemiologic and other studies related to the metabolism and action of steroid hormones are reviewed in detail. Besides hormonal factors, there are only a few established risk factors for prostate cancer. These risk factors are briefly summarized below to put the relative importance of steroid hormonal factors in perspective.

Prostate cancer incidence and mortality rates have increased in the United States over the few decades preceding the frequent use of prostate-specific antigen (PSA) for early detection (10). Even though incidence rates have increased substantially since the mid-1980s because of the use of PSA “screening” for early detection (1), incidence has declined over the period from 1990 through 1996 by an average of 2% per year and mortality by 1.6% per year (16). Because of the increasing use of PSA for

Correspondence to: Maarten C. Bosland, D.V.Sc., Ph.D., Departments of Environmental Medicine and Urology, New York University School of Medicine, 550 First Ave., New York, NY, 10016 (e-mail: maarten.bosland@med.nyu.edu).

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early detection and treatment in this period, it is too early to separately determine changes in prostate cancer rates and the impact of PSA screening on rates. In 1999, prostate cancer was the most frequent malignancy in U.S. males with 179 300 new cases expected, and it was the second most frequent cause of death as a result of cancer with 37 000 deaths expected (17).

Many studies have demonstrated that prostate cancer is more frequent in men with a family history of prostate cancer, as summarized elsewhere (10,11,15,18–20). This familial aggregation appears to be similar in African-American and in European-American men (21,22). However, inherited risk for prostate cancer can only explain a small proportion of prostate cancer cases, less than 10% (20). Besides a variety of genetic alterations identified with varying frequency in human prostate carcinomas, as summarized by Dong et al. (23), a few susceptibility loci linked to inherited prostate cancer risk have been identified on chromosome 1 (24–28) and on the X chromosome (29,30). Breast and prostate cancers cluster in some families, and there is some evidence that BRCA1 and BRCA2 mutations are involved in this clustering (18,31). However, none of these loci have thus far been associated with hormonal factors.

Evidence is limited that a history of venereal disease (11,32–34) and a history of prostatitis (34,35) are risk factors. An association between prostate cancer risk and the prior occurrence of benign prostatic hypertrophy (BPH) is biologically unlikely, even though steroid hormones are also implicated in BPH. Prostate cancer and BPH originate from different parts of the prostate (all BPH is found in the transition zone, and more than 80% of all cancers develop in the peripheral zone), and their epidemiology is dissimilar (11).

Although, in some studies (34,36,37), a relationship between smoking and risk for prostate cancer has been found, no such relationship has been observed in the vast majority of studies (37–40). In addition, smoking appears to have no effect on circulating levels of testosterone and other hormones that may be involved in prostate carcinogenesis (41,42). Most studies (11,43) addressing alcohol consumption as a potential risk factor for prostate cancer did not find evidence for an association. One notable exception is a study by Hayes et al. (44) that found a positive association in a U.S. case-control study, which was limited to heavy use of alcohol. Possible reasons for the association observed in this study are discussed by Lumey et al. (43). One of these reasons may be that prostate cancer risk is elevated in alcoholics with liver disease (11,43). This risk elevation is possibly related to the impaired clearance of estrogens described in men with liver cirrhosis (11,45,46).

Increased risk has been observed for a variety of occupations in studies of occupational factors and prostate cancer (11,14,47,48), including armed services personnel (11) and workers in the nuclear industry (11,15,48,49). Although prostate cancer risk in survivors of the atomic bomb in Japan appears not to be elevated (50), there is a rather strong international correlation between prostate cancer incidence and indoor radon levels reported (51). Thus, prostate cancer risk may be associated with exposure to ionizing radiation, but the evidence is equivocal. Associations between exposures and prostate cancer risk observed in the rubber industry are limited to one or a few plants (11). The evidence for a positive association between farming and prostate cancer risk is weak to inconclusive (11,14,47,48,52). There is only very weak, if any, evidence for an association of cadmium exposure and prostate cancer risk

(11,53,54). Hormonal factors are most likely not involved in any of these (possible) associations between risk and occupational factors.

Risk Factors Associated With Possible Hormonal Mechanisms

The results of a variety of epidemiologic studies have led to suggestions for several risk factors that may be related to a hormonal mechanism. These risks include dietary factors, vasectomy, sexual factors, the level of physical activity, and obesity.

Diet and Nutrition

The associations between dietary factors and prostate cancer risk have been extensively summarized elsewhere (10,11,15,55–57). Considerable consistency across studies indicates that a high intake of fat, particularly total fat and saturated fat, is a risk factor for prostate cancer, but the strength of the associations is modest at best (57,58) and may be greater for African-Americans than for European-Americans (59). Results from Hawaiian case-control studies suggest that as much as 25% of prostate cancer in the United States may be attributable to a high saturated-fat intake (60). However, Whittemore et al. (22) estimated that dietary fat intake may account for only 10%–15% of the difference in prostate cancer occurrence between European-Americans and African-Americans or Asians. The mechanism that could underlie an enhancing effect of fat on prostate carcinogenesis is not understood, but several hypotheses, including hormonal mediation, have been discussed elsewhere (11,15,57,61). In addition, a high intake of protein and energy and a low intake of dietary fiber and complex carbohydrates have been found to be associated with the increased risk for prostate cancer in some studies (10,11,15,55).

Associations with prostate cancer risk reported for individual nutrients or foods are not very strong. However, migration from low-risk areas, such as Japan, to high-risk countries, such as the United States, increases risk considerably (10,11). These changes in risk are thought to be due to differences in environment, including lifestyle and particularly dietary habits (10,11). It is, therefore, conceivable that the combined effects of dietary factors on prostate carcinogenesis are more important than the separate effects of any individual dietary factor (62). This idea is supported by the lack of any effect of dietary fat per se on the induction of prostate cancer in animal models, whereas epidemiologic studies (11,15,62) rather consistently show a positive association between prostate cancer risk and intake of dietary fat.

Older studies (63–67) of the effects on hormonal status of dietary changes and of the consumption of vegetarian or health food diets, which have been summarized previously (11), did not separately address the effects of dietary fat. However, they clearly indicate that diet can influence circulating hormone levels by changing androgen production rates and/or the metabolism and clearance of androgens and estrogens. In a study reported by Dorgan et al. (68), controlled changes in fat and fiber were applied to healthy men. The combination of a high-fat, low-fiber diet increased both total testosterone (by 13%) and testosterone bound to sex hormone-binding globulin (SHBG; by 15%) in the plasma as well as urinary testosterone excretion (13%), compared with a low-fat, high-fiber diet. However, urinary excretion of estrone, estradiol, and the 2-hydroxy metabo-

lites of these estrogens was lower. All of these studies indicate that diet can affect steroid hormone status, but no studies have addressed the separate effects of single dietary factors, such as fat intake.

Complete consistency is lacking among epidemiologic studies of prostate cancer risk and intake of dietary vitamin A and β -carotene (10,11,15,69). It is possible that retinoids and/or carotenes enhance rather than inhibit prostatic carcinogenesis under certain circumstances or in certain populations (69), although animal and *in vitro* studies suggest a protective effect of retinoids (11). In two more recent experiments on prostate cancer chemoprevention in a rat model, 9-*cis*-retinoic acid, a major retinol metabolite in mammalian species, strongly inhibited the induction of prostate cancer (70), but *N*-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid, did not have any effect (71). 9-*cis*-Retinoic acid is unique in that it is a pan-agonist for retinoic acid receptors, binding both retinoic acid receptor (RAR) and retinoid X receptor (RXR) receptors. *In vitro*, however, both 9-*cis*-retinoic acid and 4-HPR inhibit the growth and induce apoptosis of the androgen-sensitive human prostate cancer LNCaP cell line, and so does all-*trans*-retinoic acid, which only binds to RAR (72–74). There are indications that 4-HPR acts via a nonreceptor mechanism (74). The specific mechanism is not known by which retinoids and/or carotenoids may inhibit or enhance prostate carcinogenesis, but inhibition seems biologically more plausible than enhancement, as discussed previously (11). The retinoic acid and androgen receptors both belong to the steroid receptor superfamily (75). This circumstance raises the intriguing possibility that retinoids may be able to bind to and activate mutated forms of the androgen receptor or that the retinoic acid RAR and/or RXR may activate transcription of androgen-regulated genes. Studies on the regulation by sex steroids and retinoic acid of glutathione *S*-transferase in hamster smooth muscle tumor cells (76) and on androgen-receptor gene expression in human breast cancer cells (77) suggest that such mechanisms may exist.

Vasectomy

Vasectomy has been identified as a possible risk factor for prostate cancer in seven case-control studies (34,78–83) and in three cohort studies (84–86). The range of risk ratios in the case-control studies was 1.4 to 5.3. No elevation of risk for prostate cancer following vasectomy was found in six other case-control studies (87–92) and in two retrospective cohort studies (93–95). Although a meta-analysis (96) of 14 studies indicated that there is no causal relation between vasectomy and prostate cancer, further studies, particularly cohort studies, will be required to definitively establish whether or not vasectomy is a true risk factor for prostate cancer (58,97–99).

Three mechanisms by which vasectomy could enhance risk have been proposed: elevation of circulating androgens, immunologic mechanisms involving antisperm antibodies, and reduction of seminal fluid production (34,78,79,85,90,98,100). Most studies (101–105) that investigated the effect of vasectomy on pituitary–gonadal function did not find any effect, but some studies (90,100,106–110) found slight, but statistically significant, changes in circulating levels of certain hormones. Four groups (34,100,108,111) reported slightly elevated circulating testosterone levels, but only in two of these groups (100,108) was the increase statistically significant. Mo et al. (100) also found elevated levels of 5 α -dihydrotestosterone (DHT), the ac-

tive metabolite of testosterone in the prostate, in vasectomized men. John et al. (90) reported a decrease in SHBG, and Honda et al. (34) observed an increase in the ratio of testosterone to SHBG. These results suggest an elevation of circulating free testosterone following vasectomy, which may be a critical factor associated with risk for prostate cancer. A possible specific mechanism whereby vasectomy could influence the hypothalamic–pituitary–gonadal axis is not known.

Sexual Factors

Attempts have been made in several case-control studies (11,32–34,112–114) to investigate the possibility that sexual factors play a role in prostate cancer etiology. The results of these studies suggest that prostate cancer risk may be associated with the level of sexual activity, but no conclusive evidence exists for such a relation (11). Tsitouras et al. (115) reported a significant positive association between the level of sexual activity (intercourse and masturbation) and circulating total testosterone levels in men between the ages of 60 and 79 years as well as an absence of a decrease in testosterone levels with aging in sexually active men. These findings suggest that a hormonal mechanism may underlie a possible association between prostate cancer risk and sexual activity suggested by the aforementioned case-control studies.

Physical Activity and Anthropometric Correlates of Risk

Contradictory indications are found that the level of physical activity may be a possible risk factor for prostate cancer, but the evidence for such an association is inconclusive at present (15,62,116). Sports exercise may decrease, as well as increase, circulating androgen concentrations or have no effect, depending on such factors as the time of blood sampling in relation to the exercise, the level of exercise, and the training protocol followed (117,118). Therefore, it is possible that the type and extent of physical activity influence circulating androgen concentrations and, thereby, perhaps prostate cancer risk. At present evidence is contradictory that obesity or an increased body mass index is a risk factor for prostate cancer (15,62,119). Severson et al. (120) observed a significant increase in prostate cancer risk with increasing upper-arm circumference and upper-arm muscle area but not fat area. A positive association between prostate cancer risk and muscle mass, but not fat mass, may suggest exposure to endogenous or exogenous androgenic hormones or other anabolic factors (120,121). Indeed, evidence is available that body mass index is inversely associated with plasma testosterone and SHBG levels and positively associated with estradiol levels (119,122,123), as discussed elsewhere (11,42).

Epidemiologic Studies of Endogenous Hormones and Hormone Metabolism

As indicated earlier, a causal relation between androgens and prostate cancer development is generally considered biologically plausible because this malignancy develops in an androgen-dependent epithelium and is usually androgen sensitive. In addition, a few case reports (124–129) are available of prostate cancer in men who used androgenic steroids as anabolic agents or for medical purposes, suggestive of a causal relationship.

Studies (11,15,130) comparing the endocrine status of human prostate cancer patients with that of control subjects are probably not very informative about the endocrine status prior to the

onset of the disease and are, therefore, not meaningful to explore this relationship; in addition, the presence of the malignancy may by itself alter hormonal status. Indeed, the results of such studies do not provide a consistent pattern as summarized by Andersson et al. (130), which is confirmed in other case-control studies (119,131–133). These types of studies will, therefore, not be discussed here.

Nested case-control studies of steroid hormonal factors in ongoing cohort studies, as well as studies comparing healthy males in populations that are at high risk for prostate cancer with populations at lower risk, are likely to be more meaningful. These studies are summarized in the following sections. The two major hypotheses for these studies were that increased risk for prostate cancer would be associated with either an increased testicular production of testosterone or an increased conversion of testosterone to DHT because of an increased 5 α -reductase activity (134–136). Studies have focused on the notion that functional genetic polymorphisms in the 5 α -reductase gene or in genes involved in testosterone biosynthesis (the CYP17 gene) or DHT catabolism (the 3 β -hydroxysteroid dehydrogenase gene) could be responsible for increased testosterone production or increased DHT levels (136). In addition, polymorphisms have been discovered in the androgen receptor gene that can have functional significance for androgen receptor activity (137–139). Such polymorphisms have been postulated to be critical determinants of prostate cancer risk at the population or individual level by affecting intraprostatic DHT concentrations and androgen receptor transactivation (18,136).

Steroid Hormonal Factors in Populations That Differ in Risk For Prostate Cancer

Circulating of steroid hormone levels. A summary of the results of studies that compared circulating levels of steroid hormones in very high-risk African-Americans with those in high-risk European-Americans, lower-risk Asian-Americans, and very low-risk Asians living in Asia or African black men is provided in Table 1. The details of each study are summarized in the following paragraphs.

Ahluwalia et al. (140) studied 170 African-Americans and 55 black-Nigerian men who were matched control subjects in a case-control study of prostate cancer and were older than 50 years. Plasma levels of testosterone and estrone were significantly higher in the African-American men than in the Nigerian men, whereas levels of DHT and estradiol were not different. Similar differences were found for the prostate cancer case patients.

Hill et al. (63–65) compared the hormonal status of small groups ($n = 11$ –20) of 40- to 55-year-old African-American, European-American, and black (rural) South African men consuming their customary diets (the effects of diet changes were also studied in these men; see earlier section on “Diet and Nutrition”). In a separate study (141), African-American, European-American, and black South African boys (ages 12–15 years) and young African-American and black South African men (ages 18–21 years) were compared. In the older men, plasma levels of the testosterone processor dehydroepiandrosterone (DHEA) were significantly lower in the two groups of black men than in the white men, whereas estrone levels were higher. Plasma levels of the testosterone processor androstenedione and estradiol were significantly higher in the African men than in the two American groups, whereas no differences were

noted among these groups in testosterone levels. In the study with the 12- to 15-year-old boys and young men, similar findings were obtained for testosterone and DHEA. However, androstenedione levels were significantly lower (not higher) in the African than in the American subjects, and estradiol was lower in young black boys (12–14 years old) than in white boys but higher in older black boys (12–14 years old) and young black men than in white boys and men. These data suggest a complex interaction between ethnic background and environmental differences that change over the years of sexual maturation. In these studies by Hill and colleagues among South African black men, the 18- to 21-year-old men were different from those in 40- to 55-year-old men for androstenedione and DHEA. This divergence suggests that it is probably important for the interpretation of hormonal profiles to separately consider younger and older men.

Ross et al. (134) compared 50 healthy young African-American men (at very high risk for prostate cancer) and 50 young European-American males (at half the risk of the black men). Total circulating testosterone was 19% higher, and free testosterone was 21% higher in the group of black subjects than in the group of white subjects. Serum estrone concentrations were also significantly higher (16%) in the black than in the white group. No significant differences were seen between the groups in circulating estradiol and SHBG levels. The authors estimated that the 19%–21% difference in circulating testosterone is sufficiently large to explain the twofold difference in prostate cancer risk between white and black men in the United States. This study suggests an association between prostate cancer risk and high concentrations of circulating androgens and, possibly, estrogens.

Henderson et al. (142) compared circulating hormone levels in 20 pregnant African-American with 20 European-American women in their first trimester. Serum testosterone levels were 47% higher in black women than in white women, and estradiol levels were 37% higher. No significant differences were observed in circulating SHBG and human chorionic gonadotrophin, or in relevant pregnancy characteristics, such as the sex ratio of the offspring. These findings suggest that African-American males are exposed to higher androgen concentrations than European-American males even before birth.

The U.S. black and white men from the study by Ross et al. (134) were compared with 54 Japanese men of the same age (mean age, 19–23 years) in a follow-up study (135). The serum testosterone levels of Japanese men were not lower than those of the U.S. whites and blacks but were intermediate between these two groups, whereas their SHBG levels were significantly lower. This finding may suggest a higher percentage of free testosterone in the Japanese (at very low risk) than in the U.S. men (at high risk), but free testosterone was not measured. Compared with the Japanese men, the two U.S. groups had significantly higher circulating levels of the conjugated androgen metabolites androsterone glucuronide (41%–50% higher) and 3 α ,17 β -androstenediol glucuronide (25%–31% higher). This finding suggests that, in comparison with the high-risk U.S. groups, the low-risk Japanese population has a lower testosterone metabolism, most likely a lower activity of the enzyme 5 α -reductase that converts testosterone to DHT and the testosterone precursor androstenedione to androsterone. However, the markedly higher levels of androsterone glucuronide in U.S. men could also be indicative of a higher testosterone production in comparison with Japanese men.

Table 1. Summary of 11 studies of circulating steroid hormone levels (given as percentage of a referent group) in men from different ethnic groups*

Hormone	Study†	Mean age‡ (range, y)	African- American	European (American)	Asian-American		Asian		(South) African black men
					Japanese	Chinese	Japanese	Chinese	
Testosterone	1	≥50 y	100						Lower§
	2	43–49 (40–55)	108	100					108
	3	12–15	Same	100					Same
		18–21		100					Same
	4	20 (18–22)	118.6§	100					
	5	20, 19, 23¶	111.3	100	104.7				
	6	25 (18–47)		100				Same	
	7	63, 70 (50–79)		100			85.7§		
	8	38 (30–50)	103.5	100		101.6			
	9	70 (35–89)	105.2	100	110.5§	108.8§			
	10	20–39		100		Higher		Lower#	
Free testosterone	4	20 (18–22)	121.2§	100					
	6	25 (18–47)		100				~100	
	9	70 (35–89)	104.0	100	106.7	112.0§			
	10	20–39		100		Same		Same	
% Free testosterone	4	20 (18–22)	103.2	100					
Bioavailable testosterone	6	25 (18–47)		100				Same	
	9	70 (35–89)	103.3	100	105.1	111.4§			
SHBG	4	20 (18–22)	104.8	100					
	5	20, 19, 23¶	109.2	100	71.4§				
	7	63, 70 (50–79)		100			115.7§		
	9	70 (35–89)	106.0	100	107.8§	96.6			
	10	20–39		100		Higher		Same#	
Dihydrotestosterone (DHT)	1	≥50 y	100						Same
	6	25 (18–47)		100				112.6	
	7	63, 70 (50–79)		100			89.0		
	9	70 (35–89)	107.0§	100	107.2§	99.1			
DHT/testosterone ratio	7	63, 70 (50–79)		100			107.0**		
	9	70 (35–89)	102.0	100	96.0	89.9§			
Androsterone–glucuronide	5	20, 19, 23¶	93.8	100	66.6§				
	6	25 (18–47)		100				59.8§	
Androstanediol–glucuronide	5	20, 19, 23¶	95.4	100	76.4§				
	6	25 (18–47)		100				56.8§	
DHEA	2	43–49 (40–55)	76.9§	100					67.6§
	3	12–15	Same to lower§	100					Much lower§
		18–21		100					Same
DHEA–sulfate	9	25 (18–47)		100				68.4§	
	11	≥50¶	83.8–99.4	100					
Androstenedione	2	43–49 (40–55)	96.8	100					128.6§
	3	12–15	Same to higher§	100					Much lower§
		18–21		100					Lower§
	6	25 (18–47)		100				75.6§	
Estrone	1	≥50 y	100						Same
	2	43–49 (40–55)	117.4§	100					150.8§
	4	20 (18–22)	116.1§	100					
Estradiol-17β	1	≥50 y	100						Same
	2	43–49 (40–55)	103.1	100					128.3§
	3	12–14	Lower§	100					Same to lower§
		15	Higher§	100					Higher§
		18–21		100					Higher§
	4	20 (18–22)	109.8	100					
	7	63, 70 (50–79)		100			87.0§		

*Values are presented as the percentage of the value (set at 100%) in a referent group [European(-American) group, except in study 1, in which African-Americans are used as the reference group]. A two-sided *P* value of less than .05 was considered significant. Unless designated, none of the other differences are statistically significant.

†Studies: 1) Ahluwalia et al. (140); 2) Hill and colleagues (63–65); 3) Hill et al. (141); 4) Ross et al. (134); 5) Ross et al. (135); 6) Lookingbill et al. (143); de Jong et al. (144); 8) Ellis and Nyborg (145); 9) Wu et al. (146); 10) Santner et al. (148); 11) Corder et al. (147).

‡Mean (occasionally median) age (in years) is given with the range (if available) in parentheses. For studies 5 and 7, the mean age is given for each ethnic group consecutively. If mean age is not available, only the age range is given.

§Statistically significantly different from the European(-American) group (referent).

||If exact numerical information is not available, significant higher or lower values are indicated as such and absence of significant differences are indicated by "same."

¶Exact age range is not available.

#Significantly different from Chinese-Americans.

**Significant only after age adjustment.

Lookingbill et al. (143) reported a similar observation, comparing 53 normal healthy U.S. Caucasians and 57 Chinese males in Hong Kong between the ages of 24 and 26 years. The Caucasian men had 67% higher serum levels of androsterone glucuronide and 76% higher levels of $3\alpha,17\beta$ -androstenediol glucuronide than the Chinese men did. Circulating levels of testosterone, free testosterone, or DHT were not significantly different, but Caucasian men had 46% higher serum levels of the androgen precursor DHEA sulfate and 32% higher levels of androstenedione. These data are also suggestive of a higher 5α -reductase activity in high-risk Caucasians than in low-risk Chinese men, and they suggest an increased production of androgen precursors in the Caucasians.

In contrast to the observations of Ross et al. (134,135) and those of Lookingbill et al. (143), De Jong et al. (144) found 71% higher circulating total testosterone levels in 123 Caucasian-Dutch men (high risk) than in 91 Japanese men (low risk). The men in these studies were considerably older (50–79 years) than those studied by the previous two other groups. DHT levels were not different, but the ratio of DHT to testosterone was 10% lower in Dutch men than in Japanese men, possibly reflecting lower 5α -reductase activity; no data were presented on androgen metabolites. Serum levels of estradiol were 15% higher (significant) in the Dutch men than in the Japanese men. SHBG levels were not different, but the ratio of testosterone to SHBG concentrations was 34% higher in Dutch men than in Japanese men, which suggests higher amounts of free testosterone in Dutch men, but this parameter was not measured separately.

Ellis and Nyborg (145) studied 4462 U.S. Army Vietnam veterans, ages 31–50 years, and compared serum testosterone levels in 3654 non-Hispanic white men (mean 6.37 ng/mL) with those in 525 African-Americans (6.58), 200 Hispanics (6.33), 34 Asian/Pacific Islanders (6.89), and 49 Native Americans (6.31). The serum testosterone levels in the African-American men were significantly higher than those in the non-Hispanic white men, but the differences among the other groups were not significant. The serum testosterone difference between black and white men was larger in men between 31 and 35 years of age (6.6%) than for men ages 35–40 years (3.7%) or ages 40–50 years (0.5%). No other hormones were measured in this study.

Wu et al. (146) conducted a population-based study, comparing circulating hormone levels in 1127 healthy men: 325 African-American men, 411 European-American men, 126 Chinese-Americans, and 275 Japanese-Americans with a median age of 69.6 years (range, 35–89 years), 8.2% of whom were 60 years or younger. Serum levels of total testosterone were slightly, but significantly, higher (9%–11%) in Asian-Americans than in European-American men, whereas they were intermediate and not significantly different from the two other groups in African-Americans. The same pattern was found for serum levels of bioavailable testosterone (not bound to SHBG) and the percentage of free testosterone (not bound to either SHBG or albumin), but only the 11%–12% difference between Chinese-American and European-American men was significant. SHBG levels were not different among the four groups. In comparison with European-American men, DHT levels were 7% higher (significant) in high-risk African-Americans and low-risk Japanese-Americans, but similar in Chinese-Americans. The ratio of DHT to testosterone was 10% lower (significant) in Chinese-Americans than in European-Americans, but not significantly different in African-Americans and Japanese-Americans who had slightly higher

and lower ratios, respectively, than European-Americans. These data do not appear to provide clear support for the notions of a relation between increased 5α -reductase activity or testosterone production and prostate cancer risk, but this study did not include more direct indicators, such as androsterone glucuronide and $3\alpha,17\beta$ -androstenediol glucuronide.

DHEA sulfate was measured by Corder et al. (147) in stored serum samples of 90 African-American and 91 European-American men with prostate cancer and equal numbers of matched controls who were identified in a nested case-control study in a cohort of men in the Kaiser Permanente Medical Care Program in Northern California. Regardless of age, no significant differences were found between the two groups in DHEA sulfate levels, which were lower in men 57 years and older than in younger men.

Santner et al. (148) conducted the only study to date in which androgen production and metabolism by 5α -reductase were determined in a direct fashion in populations with different risk for prostate cancer. A radioisotope method involving intravenous administration of tritiated testosterone was used to measure the conversion of testosterone to DHT in healthy European-Americans (ages 22–27 years), Chinese-Americans (ages 20–37 years), and Chinese men living in Beijing, China (ages 24–39 years). No differences in conversion of testosterone to DHT were found among these three groups. Circulating testosterone and SHBG levels were lower in the Beijing Chinese than in the two U.S. groups, and the differences with the U.S. Chinese subjects were significant, whereas no differences were found in free testosterone. There was a nonsignificant trend toward lower calculated metabolic conversion rates of testosterone comparing European-Americans with the Chinese groups and U.S. Chinese with Beijing Chinese. Calculated testosterone production rates were lower in Beijing Chinese than in the two U.S. groups, the difference with American Chinese being significant. The ratios of urinary 5β - to 5α -reduced steroids, which are an indicator of overall 5α -reductase activity, were also not different in 20 European-American male students compared with 20 Chinese students living in Hong Kong. Urinary excretion of androsterone, etiocholanolone, and total ketosteroids was lower in the Chinese than in the U.S. students, which was significant when the data were combined with those of 20 female students from each of the two populations. Taken together, these data indicate that 5α -reductase activity is not different in Asian and Caucasian men and is not affected by the environment in which Asian men live. However, these results suggest that the living environment influences testosterone production in Asian men.

Polymorphisms in genes involved in steroid hormone metabolism and action. Studies have addressed the hypothesis (137–139) that functional polymorphisms in the 5α -reductase gene, in genes involved in testosterone biosynthesis or DHT catabolism, and in the androgen receptor gene could be associated with the differences in prostate cancer risk among various populations. These studies are summarized in the following paragraphs.

The SRD5A2 gene, which encodes for human type II 5α -reductase enzyme, is expressed in the prostate and is located on chromosome 2p23 (149,150) and contains polymorphic TA dinucleotide repeats in its transcribed 3' untranslated region (151). Reichardt et al. (152) demonstrated that TA(0) [87 base pairs (bp)] is the most common allele and was homozygous in 81% of non-Hispanic, white Americans ($n = 68$), 78% of Asian-

Americans ($n = 37$), and 67% of African-Americans ($n = 94$). The next most common allele TA(9) (103–105 bp) is heterozygous with the TA(0) allele and occurred in 19% of the non-Hispanic, white American men, 22% of the Asian-Americans, and 15% of the African-Americans. The TA(18) allele (212–131 bp) was only found in African-Americans (18%) as heterozygous with the TA(0) allele in all except one who was homozygous. Thus, the longer alleles are unique to African-Americans and may be related to their extremely high risk for prostate cancer. However, the functional significance of these polymorphisms is not yet known.

Makridakis et al. (153) identified another polymorphism in the SRD5A2 gene, the presence of a valine to leucine mutation at codon 89. If this mutation occurs in a homozygous state, it confers 28% lower 5 α -reductase activity as measured in Asian men with this genotype compared with heterozygous men and men without the mutation. These researchers observed that the frequency of the 89 valine–valine genotype was 59% in African-American men ($n = 95$), 57% in non-Hispanic white Americans ($n = 49$), 48% in Latino Americans ($n = 40$), and 29% in Asian-Americans ($n = 102$). The 89 valine–leucine genotype occurred in 37%–39% of African-Americans, non-Hispanic white Americans, and Latino Americans, and in 49% of Asian-Americans. The frequency of the 89 leucine–leucine genotype (lower 5 α -reductase activity) was 3%–4% in African-American and non-Hispanic white Americans, 15% in Latino Americans, and 22% in Asian-Americans. A recent report from another, larger study by Lunn et al. (154) is essentially consistent with these findings. In this study, the frequency of the 89 valine–valine genotype was 65% in African-American men ($n = 118$), 41% in European-Americans ($n = 176$), and 15% in Asians (Taiwanese) ($n = 108$). The 89 valine–leucine genotype occurred in 32% of African-Americans, 50% of European-Americans, and in 57% of Asians. The frequency of the 89 leucine–leucine genotype (lower 5 α -reductase activity) was 2.5% in African-Americans, 8.5% in European-Americans, and 28% in Asian-Americans. The higher frequency of the 89 leucine–leucine genotype in Latino American men and particularly Asians may be related with the lower risk for prostate cancer found in these two ethnic groups, and the low frequency of 86 leucine alleles in African-Americans may be related to their extremely high risk. However, there appears not to be a relation between plasma concentrations of 3 α -androstenediol-glucuronide as an indicator of 5 α -reductase activity and the three different SRD5A2 gene codon 89 genotypes (155).

Makridakis et al. (156) also identified another polymorphism in the SRD5A2 gene, a mis-sense alanine to threonine mutation at codon 89. An *in vitro* construction of the mutant enzyme displayed a substantial increase in activity (V_{\max}). The frequency of the mutation was very low, 1.0% and 2.3%, in healthy, high-risk African-Americans and lower-risk Hispanic men, respectively. Although no data were presented on other ethnic/racial groups, it seems unlikely that this mutation is responsible for the large ethnic/racial variations in prostate cancer risk.

The CYP17 gene, which encodes for the cytochrome P450C17 α enzyme that has both 17 α -hydroxylase and 17,20-lyase activity in the adrenal and testicular biosynthesis of androgens, is located on chromosome 10q24.3 (157). This gene is polymorphic with two common alleles, the wild-type CYP17A1 allele and the CYP17A2 allele containing a single base pair

mutation in the untranscribed 5' region of exon 1 (157). This mutation creates an additional Sp1 site in the promoter region, suggestive of increased expression potential (157). The functional significance of this polymorphism in men is not known, but premenopausal and postmenopausal women with the A2 allele have been reported to have higher circulating estradiol and progesterone levels than women homozygous for the A1 allele (158,159). Circulating levels of DHEA and androstenedione, but not testosterone, were increased in postmenopausal women (159). Lunn et al. (154) recently reported that the frequencies of the A1/A1 and A1/A2 genotype were between 40% and 44%, and the frequency of the A2/A2 genotype was 16%–17% in both African-American men ($n = 115$) and European-Americans ($n = 115$), accounting for an A2 allele frequency of 0.36–0.38. In Asians (Taiwanese; $n = 110$), however, the A1/A1 genotype occurred in 24%, the A1/A2 genotype in 49%, and the A2/A2 genotype in 27%, with an A2 allele frequency of 0.52. The frequency differences between the Asians and the two American groups were statistically significant and are perhaps related to the low risk for prostate cancer in Asian men.

Verreault et al. (160) reported complex dinucleotide polymorphisms in the 3rd intron of the human HSD3B2 gene, located on chromosome 1p13, which encodes type II 3 β -hydroxysteroid dehydrogenase, which is expressed in the adrenals and testes, and catabolizes DHT (161). Devgan et al. (162) reported that the frequency of HSD3B2 alleles differs between African-American, European-American, and Asian men. One minor allele is unique for African-American men (6% allele frequency), whereas the most common allele is more frequent in European-Americans (52%) than among African-American or Asian men (34%–37%). The second most common allele is more frequent in African-Americans (25%) than in either Asians (15%) or European-American men (11%). As with the TA dinucleotide polymorphisms in the SRD5A2 gene, the functional significance of these HSD3B2 gene polymorphisms is not known.

The human androgen receptor gene, which is located on the X chromosome, also contains polymorphisms that are found as 8–31 CAG and 8–17 GGC (or GGN) microsatellite repeats in exon 1 encoding for the N-terminal domain of the protein where transactivation activity resides (139). The CAG repeat length has been demonstrated to determine transactivation activity of the androgen receptor, with 40 or more repeats being associated with human androgen insensitivity syndromes, such as spinal and bulbar muscular atrophy, and reduction of repeat length leading to increased transactivation activity *in vitro* (137–139). The functional significance of the GGC repeat length is not clear. Irvine et al. (163) reported that 75% of African-Americans ($n = 44$) had CAG repeat lengths of less than 22, whereas 62% of European-Americans ($n = 39$) and 49% of Asian-Americans ($n = 39$) had such shorter alleles. Very short alleles (<17 CAG repeats) occurred almost exclusively in African-Americans. The most common GGC allele (16 repeats) was found in 70% of Asian-Americans, 57% of European-Americans, and only 20% of African-Americans. The frequency of short GGC repeats (<16) was 61% in African-Americans, 27% in Asian-Americans, and 11% in European-Americans. GGC repeats longer than 16 were rare in the Asian-American men (3%) but more frequent in African-Americans (20%) and European-Americans (32%). Sartor et al. (164) essentially confirmed the findings on CAG repeats in a sample of African-Americans ($n = 65$) and European-American men ($n = 130$). Mean and median number of CAG

repeats was 19 in African-Americans and 21 in European-Americans, and 57% of the African-American men had less than 20 repeats, whereas only 28% of European-American men had such short repeats. Ekman et al. (165), however, did not find significant differences in the distribution of CAG repeats comparing Swedish and Japanese men with BPH but without cancer ($n = 38$ and 33 , respectively), but Swedish men with prostate cancer ($n = 118$) had somewhat shorter CAG repeats (mean, 15.9; median, 15) than Japanese prostate cancer patients ($n = 34$; mean, 17.5; median, 17). In conclusion, in two studies short CAG repeat alleles in the androgen receptor gene, which are probably associated with greater androgen receptor transactivation activity, were most frequent in the highest-risk population (African-Americans) and least frequent in the lowest-risk group (Asian-Americans), whereas the frequency was intermediate in intermediate-risk European-Americans. The high frequency of short GGC repeats found in African-Americans may also be related with their extremely high risk for prostate cancer, but the functional significance of this polymorphism is not yet known.

Summary and conclusions. When examining Table 1, few clear or convincing patterns emerge about associations between circulating hormone concentrations and prostate cancer risk at the population level. Two studies examined levels of androstosterone glucuronide and $3\alpha,17\beta$ -androstenediol glucuronide, which are considered (166–168) indicators of 5α -reductase activity, particularly $3\alpha,17\beta$ -androstenediol glucuronide, which is a direct metabolite of DHT. In both studies, the levels of these 5α -reduced androgen metabolites were lower in low-risk Asian populations than in high-risk European-Americans (135,143). These findings suggest lower 5α -reductase activity in the Asians and consequently reduced formation of DHT and androgenic stimulation of the prostate. This notion is supported by the reported higher frequency in Asians than in European-Americans or African-Americans of a polymorphism in the 5α -reductase (SRD5A2) gene that appears to be associated with lower 5α -reductase activity (a valine to leucine mutation at codon 89) (136,153). However, no differences were found between Asians and European-Americans in a study in which overall conversion of testosterone into DHT was directly measured (148). Furthermore, androstosterone glucuronide and $3\alpha,17\alpha$ -androstenediol glucuronide levels were not higher in very high-risk African-Americans than in intermediate-risk European-Americans, and circulating levels of DHT and the ratio of DHT to testosterone were not different in ethnic populations (Asian, black, and white) that differ in prostate cancer risk (143,144,146). Thus, the relation between 5α -reductase activity and prostate cancer risk at the population level remains unclear at present.

Circulating levels of testosterone and/or free testosterone were slightly higher in African-Americans than in European-Americans in five of six studies that examined this question, but this finding is statistically significant in only one study (134). Furthermore, lower as well as higher testosterone concentrations have been found in lower-risk Asian or African men compared with European-Americans or African-Americans, although testosterone levels were lower in Asians living in Asia than in American populations regardless of ethnicity in two of three studies. Thus, these studies in ethnic/racial groups provide at present no substantive evidence in support of the hypothesis of a causal positive relation between elevated 5α -reductase activity and prostate cancer risk at the population level and only very

limited evidence for elevated (free) testosterone levels being associated with prostate cancer risk.

The only other patterns appearing in the data in Table 1 are that levels of estrogens are slightly higher (in five of five studies) and those of DHEA (sulfate) lower (in three of three studies) in black Africans and African-Americans than in men of European descent—hardly any data are available on Asians in this regard (63–65,134,140,141,144). The biologic significance of these observations is unclear, but they may be related to the high susceptibility of black men to prostate cancer when they live in the American environment. However, the above summarized endocrine differences between very high-risk African-Americans and high-risk European-Americans were not consistent in younger and older men, and they were not similar to the differences observed between the high-risk U.S. populations and the low-risk African black men (63–65,140,141). These inconsistencies raise the possibility that the factors and endocrine mechanisms that determine the difference in risk between African black men and African-Americans are dissimilar from those that determine the risk difference between African-Americans and European-Americans (11).

Finally, CAG repeat length polymorphism in the androgen receptor gene was found to be associated with prostate cancer risk in two studies. Short CAG repeat alleles are probably associated with greater androgen receptor transactivation activity. Such short CAG repeat alleles were most frequent in African-Americans (very high-risk), least frequent in Asian-Americans (low risk), and intermediate in European-Americans (intermediate risk). Another androgen receptor polymorphism in GGC repeats may also be related with risk for prostate cancer, but the functional significance of this polymorphism is unknown.

Association of Steroid Hormonal Factors With Prostate Cancer Risk in Population-Based Case-Control Studies

Circulating of steroid hormone levels in nested case-control studies. A summary of the results of population-based, nested case-control studies that examined the association between circulating levels of steroid hormones and risk for prostate cancer is provided in Table 2. The details of each study are summarized in the following paragraphs. One study by Carter et al. (169) concerned only 16 case subjects and contained considerable bias because of storage effects on hormone measurements, which were recognized but not controlled for. This study is, therefore, not further discussed here.

Nomura et al. (170) compared 98 prostate cancer case patients with matched control subjects from a cohort of 6860 Hawaiian-Japanese men, which were followed for an average of 14 years. No significant differences were found between case patients and control subjects or associations with risk for serum levels of testosterone, DHT, estrone, estradiol, and SHBG, measured once at the start of the cohort study (free testosterone levels were not determined). An elevation in risk was only observed for an increasing ratio of testosterone to DHT, which was borderline significant. This latter observation perhaps suggests an inverse relation between (peripheral) 5α -reductase activity and prostate cancer risk.

Barrett-Connor et al. (171) followed a Californian cohort of 1008 white, upper-middle class men between the ages of 40 and 79 years for a period of 14 years, during which time 57 cases of prostate cancer occurred (26 deaths and 31 incident cases). No significant relation was found between the risk for prostate can-

cer and baseline serum concentrations of testosterone, estrone, and SHBG. However, RR increased linearly with an increasing serum level of androstenedione, a testosterone precursor. RR also increased linearly with an increasing serum level of estradiol, but this finding was not statistically significant.

Hsing and Comstock (172) and Comstock et al. (173) reported results of a population-based, nested case-control study in a cohort of 25 620 men (98% European-American) in Maryland. Blood samples were obtained in 1974, and 98 cases of prostate cancer were identified in the first 13 years of follow-up (81 cases in 12 years of follow-up for DHEA and DHEA sulfate). Men 70 years and older as well as men younger than 70 years were studied separately (except for DHEA and DHEA sulfate). No significant differences were found between case patients and control subjects or associations with risk for baseline serum testosterone, DHT, DHEA, DHEA-sulfate, estrone, or estradiol. The ratio of testosterone to DHT was higher in case patients than in control subjects of all ages, and, for men younger than 70 years but not for older men, risk for prostate cancer increased with an increasing testosterone/DHT ratio; both findings were borderline significant ($0.05 < P < 0.1$). This latter observation could suggest an inverse relation between (peripheral) 5 α -reductase activity and prostate cancer risk.

Nomura et al. (174) reported a follow-up of their 1988 study, including 141 case patients and 141 matched control subjects from their cohort of 6860 Hawaiian-Japanese men followed for an average of more than 20 years. In this population-based, nested case-control study, there were no significant differences between case patients and control subjects or associations with risk for baseline serum testosterone, free testosterone, DHT, ratio of testosterone to DHT, androsterone-glucuronide, 3 α -androstanediol-glucuronide, and androstenedione.

DHEA sulfate was measured by Corder et al. (147) in stored serum samples of 181 men with prostate cancer (90 African-Americans and 91 European-Americans) and equal numbers of matched control subjects who were identified in a nested case-control study in a cohort of men in the Kaiser Permanente Medical Care Program in Northern California. For men younger than 57 years or for older men there were no significant differences between case subjects and control subjects in DHEA sulfate levels, which were also not associated with risk.

Gann et al. (175) conducted a prospective, nested case-control study on 222 case patients with prostate cancer and 390 matched control subjects obtained from the Physician's Health Study (a randomized intervention trial with aspirin and β -carotene in 22 071 U.S. male physicians, probably largely white), with a mean follow-up of approximately 6 years. There were no significant differences between case patients and control subjects for plasma testosterone, SHBG, DHT, ratio of testosterone to DHT, 3 α -androstanediol-glucuronide, or estradiol. Several highly significant associations were found between plasma levels of SHBG and the steroid hormones studied. Therefore, odd ratios were calculated after simultaneous adjustment for all these endocrine factors for 222 matched case-control sets. This approach resulted in significant positive associations with risk for testosterone and the ratio of testosterone to DHT and inverse associations with risk for SHBG and estradiol. A positive association was also found with risk for 3 α -androstanediol-glucuronide, which was borderline significant, but there was no association with risk for DHT. These observations support a relationship between elevated testosterone and

prostate cancer risk, but they are contradictory regarding a relation between (peripheral) 5 α -reductase activity and prostate cancer risk.

Guess et al. (176) reported on a population-based, case-control study from a cohort of more than 125 000 European-American men in the Kaiser Permanente Medical Care Program. They compared 106 case patients and matched control subjects selected from men, with a median follow-up of 14 years (range, 5–23 years). No significant differences were found between case patients and control subjects or associations with risk for baseline serum testosterone, free testosterone, DHT, androsterone-glucuronide, or 3 α -androstanediol-glucuronide.

Vatten et al. (177) conducted a population-based, nested case-control study of 59 case patients with prostate cancer and 180 matched control subjects from a cohort of approximately 28 000 Norwegian men, with a mean follow-up of 10 years (range, 1–19 years). There were no significant differences between case patients and control subjects or associations with risk for baseline serum testosterone, DHT, ratio of testosterone to DHT, or 3 α -androstanediol-glucuronide.

Dorgan et al. (178) reported results from a population-based, nested case-control study of 116 case patients with prostate cancer and 231 matched control subjects from a cohort of 29 133 Finnish men from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study of cigarette smokers with a follow-up of 5–8 years. No significant differences were found between case patients and control subjects or associations with risk for baseline serum testosterone, free testosterone, SHBG, DHT, DHEA-sulfate, 3 α -androstanediol-glucuronide, androstenedione, estrone, or estradiol. There was a nonsignificant trend toward a higher ratio of testosterone to DHT in case patients than in control subjects and a positive association with risk for this ratio. This finding may suggest an inverse relation between (peripheral) 5 α -reductase activity and prostate cancer risk.

Heikkilä et al. (179) reported on the results of a population-based, nested case-control study in a Finnish cohort study in which serum was collected and stored, and a cohort of 16 481 men was followed for up to 24 years. During this period, 166 case patients with prostate cancer were identified and were matched to 300 control subjects. Serum levels of testosterone, SHBG, and androstenedione were similar in case patients and control subjects, and they were not associated with prostate cancer risk. When case patients identified in the first 8 years of follow-up were excluded, there was a borderline significant ($P = .06$) trend for increasing risk with higher levels of testosterone but not with SHBG or androstenedione. This finding supports the notion of a relationship between elevated androgen levels and prostate cancer risk.

Polymorphisms in genes involved in steroid hormone metabolism and action in population-based case-control studies. Several case-control studies have addressed the hypothesis (137–139) that functional polymorphisms in the 5 α -reductase gene, in genes involved in testosterone biosynthesis or DHT catabolism, and in the androgen receptor gene could be associated with differences in prostate cancer risk. These studies are summarized in the following paragraphs.

Kantoff et al. (180) studied the association between prostate cancer risk and the polymorphisms in TA dinucleotide repeats in the transcribed 3' untranslated region of the human SRD5A2 gene encoding for type II 5 α -reductase enzyme; the functional significance of these polymorphisms is not known, as indicated

Table 2. Summary of 10 nested case-control studies of circulating steroid hormone levels in men and their relation to risk for prostate cancer*

	Nomura et al. (170)	Barrett-Connor et al. (171)†	Hsing and colleagues (172,173)	Corder et al. (147)
No. of case patients/control subjects	98/98	57/NA†	98/98 (DHEA: 81/81)	181/181
Follow-up, y	14, approx.	14, mean	13 (DHEA: 12)	<17–24, maximum
Adjustment	—	BMI, age	—	—
Presentation values*	Mean	Median	Mean	Mean
Testosterone	97.6 OR = 0.99	105.2 RR = 1.00	102.0 OR = 1.5	
Free testosterone				
SHBG	97.5 OR = 0.85	101.4 RR = 1.04		
Dihydrotestosterone (DHT)	92.6 OR = 0.66		99.6 OR = 1.0	
Testosterone/DHT ratio	Increased** OR = 2.69§¶		94.1†† OR = 0.7 (3.0 for <70 y)	
Androsterone–glucuronide				
Androstenediol–glucuronide				
DHEA–sulfate (DHEA)			88.4 (DHEA: 89.0) OR = 0.82 (DHEA: 0.94)	96.5 OR = 1.00 (in men ≥57 y at baseline)
Androstenedione		105.2 RR = 1.26§		
Estrone (E ₁)	94.2 OR = 0.89	105.9 RR = 1.09	99.2 OR = 0.8	
Estradiol (E ₂)	95.2 OR = 0.57	107.7 RR = 1.10	105.3 OR = 1.0	

earlier. These investigators conducted a nested case-control study with the use of the Physician's Health Study cohort with 590 men with prostate cancer and 802 age-matched control subjects. They observed that the frequency of the most common genotype, TA(0)/TA(0), was 76.4% in case patients and 75.4% in control subjects, the frequency of the next most common genotypes, TA(0)/TA(9) and TA(0)/TA(18), was 22.4% in case patients and 22.1% in control subjects, and the frequency of genotypes TA(9)/TA(9) and TA(18)/TA(18) was 1.2% in case patients and 2.5% in control subjects; only two control subjects had a TA(9)/TA(18) genotype. Men that were homozygous for long repeats, TA(9)/TA(9) or TA(18)/TA(18), were at lower risk for prostate cancer than men with the predominant TA(0)/TA(0) genotype, with a borderline significant ($P = .08$) odds ratio (OR) of 0.47. These findings sharply contrast with the aforementioned observation that the long TA(18) alleles are unique to African-Americans, who are at very high risk for prostate cancer (152).

As indicated earlier, Makridakis et al. (153) identified another polymorphism in the SRD5A2 gene, a mis-sense alanine to threonine mutation at codon 89, apparently associated with an increase in 5 α -reductase activity. In a nested case-control study conducted in the Hawaii–Los Angeles Multiethnic Cohort Study of Diet and Cancer, the frequency of the mutation appeared to be low but was responsible for 8%–9% of cases in African-

American (203 case patients and 257 unmatched control subjects) and Hispanic men (160 case patients and 193 control subjects); no data were presented on other ethnic/racial groups (153). The RR (age-adjusted) of prostate cancer for possessing a mutated allele was 3.28 (95% confidence interval [CI] = 1.09–11.87) in African-American men and 2.50 (95% CI = 0.90–7.40) in Hispanics. For advanced prostate cancer, the RRs for possessing a mutated allele were more significant: 7.22 (95% CI = 2.17–27.91) in African-American men and 3.60 (95% CI = 1.09–12.27) in Hispanics. Although the results of this study support the notion that increased 5 α -reductase activity may be related to prostate cancer risk, it seems unlikely that the alanine to threonine mutation at codon 89 in the SRD5A2 gene is involved in a substantial proportion of prostate cancer cases.

The relation between prostate cancer risk and the occurrence of the aforementioned valine to leucine mutation at codon 89 in the SRD5A2 gene, a polymorphism that is associated with reduced 5 α -reductase activity, was examined by Febbo et al. (155) and Lunn et al. (154). Febbo et al. (155) conducted a nested case-control study with the use of the Physician's Health Study cohort with 584 men with prostate cancer and 799 matched control subjects. The valine–leucine and leucine–leucine genotypes were found in 50% of case patients and 51% of control subjects and were not associated with elevated prostate cancer

Table 2 (continued). Summary of 10 nested case-control studies of circulating steroid hormone levels in men and their relation to risk for prostate cancer*

Nomura et al. (174)	Gann et al. (175)‡	Guess et al. (176)	Vatten et al. (177)	Dorgan et al. (178)	Heikkilä et al. (179)‡
141/141	222/390	106/106	59/180	116/231	166/300
>20	6.3, mean	14 (5–23)	10, mean	4.1, median	<8–24
Age at entry, date/time of blood sampling	BMI, age, smoking, alcohol, exercise, all other hormones	BMI, smoking, alcohol, diabetes	—	—	Smoking, BMI, other hormones
Mean	Median	Median	Mean	Mean	Mean
100.2 OR = 1.03	102.8 OR = 2.60§	104.7 OR = 1.00	97.1 OR = 0.83	98.2 RR = 0.8	101.6 RR = 1.23¶#
103.6 OR = 1.09		100.0 OR: 1.14		102.6 RR: 1.1	
	91.7 OR = 0.46§			96.9 RR = 0.8	100.9 RR = 1.12
103.4 OR = 0.82	94.4 OR = 0.71 0.32 for ≥62 y)		98.0 OR = 0.83	95.7 RR = 0.7	
99.0 OR = no data	102.7 OR = 2.35§		99.2 OR = 1.31	Increased** RR: 1.7	
97.4 (OR = 1.37)		101.2 (OR = 1.13)			
109.2 OR = 0.85	104.6 OR = 1.60¶	106.4 OR = 1.16	102.1 OR = 1.10	102.3 RR = 1.2	
				106.3 RR = 1.2	
104.7 OR = 1.24				99.4 RR = 1.0	92.0 RR = 0.92
				100.0 RR = 0.8	
	97.7 OR = 0.56§			100.0 RR = 1.1	

*Values presented are hormone values (means or medians) of cases as percentage of the values in controls (set at 100%) and risk estimates [as either odds ratios (OR) or relative risks (RR)] of highest tertile or quartile relative to the lowest tertile, quartile, or quintile (set at 1.00). The indicated values are for cases presented as the percentages of the values in controls (set at 100%) or risk estimates indicating the relation with prostate cancer risk. Results of statistical analysis are indicated only when significant at a (two-sided) *P* value of less than .05, considering tests for differences between the lowest (referent) and highest tertile or quartile as well as for trend.

‡This study calculated prostate cancer rates for case patients and compared them with population data, which were also used to present median hormone levels for case patients and control subjects. RRs were calculated for an increase in hormone concentration equal to 1 standard deviation.

‡The risk estimates by hormone level were adjusted for the concentrations of all other hormones considered; this adjustment was not done in any of the other studies.

§Statistically significant difference between lowest (referent) and highest tertile or quartile different from controls.

||Statistically significant trend.

¶Borderline significant trend (.05 < *P* < .1).

#This trend was borderline significant (*P* = .06) only for a follow-up of longer than 8 years.

**No exact value is available, indicates increased value compared with control subjects.

‡‡Borderline significant (.05 < *P* < .1).

risk as compared with the valine-valine genotype (OR = 0.84–0.96). Lunn et al. (154) confirmed these findings in a case-control study that employed 108 prostate cancer patients from urology clinics at the University of North Carolina and nearby Duke University. Control subjects (*n* = 156) were drawn from BPH and impotence patients at the same clinics and not matched to case patients. The groups were predominantly European-American (5%–11% were African-American). The valine-leucine and leucine-leucine genotypes were found in 56% of case patients and 49% of control subjects and were not associated with prostate cancer risk as compared with the valine-valine genotype (OR = 1.3; 95% CI = 0.8–2.2). These obser-

vations are consistent with the absence of a relation between plasma concentrations of 3 α ,17 β -androstenediol glucuronide and the three different SRD5A2 gene codon 89 genotypes reported by Febbo et al. (155).

In the same case-control study, Lunn et al. (154) also examined the association between prostate cancer risk and the aforementioned single base-pair mutation polymorphism in CYP17 gene (152c), encoding for the 17 α -hydroxylase and 17,20-lyase activity. The CYP17A2 allele containing a single base-pair mutation was found in 69% of case patients and 57% of control subjects and was associated with prostate cancer risk with an OR of 1.7 (95% CI = 1.0–3.0). The association appeared to be

limited to men younger than 65 years, with an increased OR of 2.30 (95% CI = 1.0–4.8). Contradictory findings of this association between prostate cancer risk and the presence of the CYP17A2 allele were reported from a Swedish case-control study by Wadelius et al. (181). The frequency of the A1/A2 or A2/A2 genotype was 61% in prostate cancer cases (n = 178) and 71% in population controls (n = 160). The OR of having the A1/A1 genotype versus the A1/A2 or A2/A2 genotype was 1.61 (95% CI = 1.02–2.53). This latter finding is consistent with a preliminary report of higher circulating testosterone levels found in men homozygous for the A1 allele than in men with an A2 allele of the CYP17 gene (181).

Six population-based, case-control studies with substantive numbers of cases examine the association between the aforementioned CAG and GGC (or GGN) repeat polymorphisms in the human androgen receptor gene (163,182–186). The results of these studies are summarized in Tables 3–5. As shown in Table 3, these studies indicate that CAG repeat lengths shorter than 22 may be associated with slightly increased risk for prostate cancer with elevated ORs or RRs found for <22 repeats (versus ≥22) in four studies (163,182–184), and for 20–21 repeats (versus <20 or ≥22) in one study (185). However, only in the study by Giovannucci et al. (182), but not in four other studies (163,183–185), the tendency of increased risk with decreasing repeat length was statistically significant. In addition to these six population-based, case-control studies, a study by Hakimi et al. (186) compared the CAG repeat lengths in the androgen receptor of 59 prostate cancer patients with published data of the general population (n = 370). Short repeats (≤17) were more frequent in cases than in the general population (OR = 3.7; 95% CI = 1.3–10.5; P = .02). Thus, these findings consistently indicate the possibility that prostate cancer risk is slightly increased with shorter CAG repeat alleles. This possibility may be related to a greater androgen receptor transactivation activity associated with shorter CAG repeat alleles, as indicated earlier.

Two of these six case-control studies and a seventh population-based, case-control study examined GGN or GGC repeats in the androgen receptor gene, the functional significance of which is not known at present (163,184,187). As shown in Table 4, these studies indicate that GGN or GGC repeat lengths may influence risk, but the results do not agree with one another. Hakimi et al. (186) also compared androgen receptor GGC repeat lengths in 54 prostate cancer patients with published population data (n = 110). Short repeats (≤14) were more frequent in case patients than in the general population (OR = 4.6; 95% CI = 1.3–16.1; P = .02). However, Correa-Cerro et al. (185) did not find such an association in a French-German population. Thus, even though two studies consisted of substantial numbers of case patients and control subjects (184,187), the influence of GGN or GGC repeat length on prostate cancer risk is presently not clear. The combined effects of CAG and GGN repeat length were also examined in the three case-control studies, and they were greater than those either polymorphism separately in all three, as indicated in Table 5. CAG repeats of less than 22 or 21 appeared to be the consistent factor in this interaction in all three studies and were associated with (borderline) significantly increased risk. However, the functional significance of this combined effect is not clear.

Summary and conclusions. When examining Table 2, no clear or convincing patterns emerge about associations between circulating hormone concentrations and prostate cancer risk, with few exceptions. In most studies, an association was found between increased risk and increased ratios of testosterone to DHT, which reached statistical significance in three of six studies. Although this finding suggests a relation between reduced 5α-reductase activity and prostate cancer risk, no associations were found between risk and the levels of androsterone glucuronide and 3α,17β-androstenediol glucuronide, indicators of 5α-reductase activity (166–168)—a borderline significant trend for such an association was found in only one (175) of five studies (174–178). Significant associations between prostate

Table 3. Summary of five case-control studies of CAG repeat length polymorphisms in the androgen receptor circulating levels in relation to risk for prostate cancer

Study (reference No.)	No. of case subjects	No. of control subjects	CAG repeat comparison	OR/RR	95% CI	N (case subjects/control subjects)
Irvine et al. (163)	57	39	≥22	1.00	Referent	19/15
			<22	1.25	0.88–1.73	38/24
Ingles et al. (183)	57	169	≥22	1.00	Referent	19/68
			20–21	0.89	0.41–1.94	14/56
			<20	1.91	0.94–3.88	24/45
			Trend: not significant			
Stanford et al. (184)	281	266	≥22	1.00	Referent	136/140
			<22	1.23	0.88–1.73	145/126
Giovannucci et al. (182)	587	588	≥26	1.00	Referent	60/72
			24–25	1.02	0.66–1.58	98/115
			22–23	1.17	0.76–1.80	116/119
			21	1.35	0.87–2.09	113/101
			20	1.28	0.79–2.08	69/65
			19	1.22	0.75–2.00	62/61
			≥18	1.52	0.92–2.49	69/55
			Trend: P = .04			
Correa-Cerro et al. (185)	132	105	≥24	1.00	Referent	39/28
			22–23	1.02	0.49–2.13	30/22
			20–21	1.43	0.73–2.82	34/35
			≥19	0.96	0.45–2.03	29/20
			Trend: not significant			

CI = confidence interval; OR = odds ratio; RR = relative risk.

Table 4. Summary of three case-control studies of GGC/GGN repeat length polymorphisms in the androgen receptor circulating levels in relation to risk for prostate cancer

Study (reference No.)	No. of case subjects	No. of control subjects	GGC/N repeat comparison	OR/RR	95% CI	N (case subjects/control subjects)
Irvine et al. (163)	57	37	16	1.00	Referent	30/21
			Not 16	1.18	Not presented	27/16
Stanford et al. (184)	257	250	>16	1.00	Referent	56/75
			≤16	1.60*	1.07–2.41	201/175
Platz et al. (187)	582	794	Not 23	1.00	Referent	244/369
			23	1.20	0.97–1.49	338/425

*Odds ratio (OR) or relative risk (RR) is significantly different from referent value at the $P < .05$ level. CI = confidence interval.

Table 5. Summary of three case-control studies of the interaction of CAG and GGC/GGN repeat length polymorphisms in the androgen receptor circulating levels in relation to risk for prostate cancer

Study (reference No.)	No. of case subjects	No. of control subjects	CAG/GGC-N repeat comparison	OR/RR	95% CI	N (case subjects/control subjects)
Irvine et al. (163)	57	37	≥22/16	1.00	Referent	34/28
			<22/not 16	2.10 ($P = .08$)	Not presented	23/9
Stanford et al. (184)	257	250	≥22/>16	1.00	Referent	22/32
			≥22/≤16	1.15	0.56–2.35	32/41
			<22/>16	1.54	0.83–2.86	97/93
			<22/≥16	2.05*	1.09–3.84	98/77
			Trend: $P = .008$			
Platz et al. (187)	582	794	>23/not 23	1.00	Referent	66/119
			>23/23	1.17	0.77–1.77	90/133
			21–23/not 23	1.39	0.93–2.06	75/116
			21–23/23	1.22	0.82–1.83	152/185
			<21/not 23	1.49*	1.02–2.15	103/134
			<21/23	1.62*	1.07–2.44	96/107

*Odds ratio (OR) or relative risk (RR) is significantly different from referent value at the $P < .05$ level. CI = confidence interval.

cancer risk and elevated levels of testosterone and androstenedione or decreased levels of SHBG and estradiol were found each in only a single study [(175) for testosterone, SHBG, and estradiol; (171) for androstenedione], and they were not observed in eight (testosterone), four (SHBG and estradiol), or three (androstenedione) other studies. It is possible that relevant associations may have been missed in most studies, because the data for each individual hormone were not adjusted for concentrations of other hormones studied, even though there are many intercorrelations between circulating levels of these hormones. Only in the study by Gann et al. (175) were these types of adjustments applied, after which risk was significantly associated with increased circulating testosterone levels and testosterone/DHT ratio, as well as decreased concentrations of SHBG and estradiol, and, in men older than 61 years, DHT. A meta-analysis study by Eaton et al. (188) used most but not all studies included in this overview, as well as a study that was discounted here (169) and some unpublished data. They found no significant differences for the ratios of mean hormone levels between case patients and control subjects, with the exception of slightly elevated levels of $3\alpha,17\beta$ -androstenediol glucuronide. This analysis is essentially in agreement with the analysis of this overview, with the only consistent finding slightly elevated ratios between case patients and control subjects of $3\alpha,17\beta$ -androstenediol glucuronide in five of five studies (Table 2). However, Eaton et al. (188) did not take into account the risk estimates produced by these studies, which seriously limits its conclusions.

The results of three nested case-control studies on the rela-

tion between prostate cancer risk and two different polymorphisms in the human type II 5α -reductase enzyme gene (SRD5A2) do not support the notion of an association between risk and increased 5α -reductase activity (154,155,180). However, an infrequent polymorphism associated with increased 5α -reductase activity was more common in case patients than in control subjects in one case-control study (156), which indicates that associations between polymorphisms in the SRD5A2 gene and prostate cancer risk cannot be discounted. Data on a relation between prostate cancer risk and a polymorphism in the CYP17 gene, which encodes for the 17α -hydroxylase and $17,20$ -lyase activity involved in androgen biosynthesis, are contradictory (154,181). Furthermore, the functional significance of this polymorphism in males is not yet known. In four of five similar nested case-control studies of polymorphisms in trinucleotide repeats in the promoter region of the androgen receptor gene, an association was found between risk and short CAG repeat alleles—short CAG repeat lengths are associated with greater androgen receptor transactivation activity. However, this association was weak and significant in only one study. An association between risk and polymorphisms in androgen receptor GGC or GGN repeat lengths is not clear because results of three studies were inconsistent, and the functional significance of these polymorphisms is not known. There is possibly an interaction between CAG and GGC/GGN repeat length in relation to prostate cancer risk, but results of the three studies examining this possible interaction were inconsistent. Short CAG repeats were also correlated with advanced disease and/or early onset of prostate cancer (182–186,189,190).

Taken together, the results of the above summarized studies do not provide unequivocal or strong evidence for any particular association between prostate cancer risk and circulating levels of hormones or polymorphisms in genes that encode for proteins involved in steroid hormone action or metabolism. Only a few associations with prostate cancer risk have been observed consistently (in at least three studies), and they are weak at best: 1) slightly, but mostly not significantly, higher circulating testosterone and estrogen levels and lower DHEA (sulfate) levels in high-risk African-American men as compared with lower-risk European-American men, and 2) a CAG repeat length polymorphism in the androgen receptor gene with short repeat lengths associated with increased risk and increased receptor transactivation activity. The evidence for involvement of activity of the enzyme 5 α -reductase, which is critical in androgen action in the prostate, is inconsistent and contradictory.

Difficulties in Interpretation

Several important points should be considered in interpreting these observations: First, there are numerous potential problems with most studies that measure circulating hormone levels, such as the usually large intra- and interassay variability in the immunoassays used (122,191–194). Typically, only single blood samples are available, and within-subject variations over time and possible differences in circadian rhythms cannot be taken into account. Another problem is that there are many interrelationships between various hormones (144,174), which only an occasional study has taken into account during data analysis (175). Second, young Japanese men and Chinese men from Hong Kong are probably at least partially westernized in their lifestyle (194), and they can, therefore, not simply be compared with older Asian men. Young men that are hormonally studied today may have a prostate cancer risk that is different from the currently recorded risk in older men of the same population, as suggested by the rising prostate cancer rates in Japan (194). Third, the factors that cause the differences in prostate cancer risk between black, white, and Asian men in the United States may be different from those that determine differences in prostate cancer risk between Asian or African populations and populations in the United States or West European countries, as indicated earlier.

Another crucial issue is that circulating hormone levels and polymorphisms in critical genes provide very little information about concentrations at the molecular targets of these hormones in the prostate gland or about steroid hormone metabolic processes within the prostate. For example, less than 10% of circulating DHT is produced by the prostate, and a substantial proportion of serum 3 α ,17 β -androstenediol glucuronide is derived from nonprostate sources; these two steroids are, therefore, not very good indicators of prostatic 5 α -reductase activity (166–168). Also, aromatase activity has been identified in the human prostate and the LNCaP prostate cancer cell line (195–199), although there are reports of contradictory findings (200,201) that may be related to methodologic differences. In addition, estrogen levels in the human prostate exceed those found in the circulation (202). Thus, local formation of estrogens in the prostate may occur and may contribute to dysregulation of growth. Finally, although there is some information about the functional

significance of some polymorphisms in genes encoding for proteins involved in steroid hormone action or metabolism, their influence on the prostatic activity of steroid hormone metabolizing enzymes or the activity of steroid hormone receptors in the prostate is not known. In view of the highly complex and often tissue-specific mechanisms of regulation of gene expression, it is likely that these polymorphisms have only limited and probably cell type-specific influences on these regulatory processes.

Hypotheses

From the studies summarized above, four possible hypotheses emerge about steroid hormone factors associated with prostate cancer risk: 1) slightly elevated (bioavailable) testosterone serum levels, as indicated by studies comparing healthy low- and high-risk men (134,135,140,144,148); 2) increased peripheral and possibly prostatic activity of 5 α -reductase (135,143,153,175); 3) slightly increased serum levels of estrogens, as indicated by studies comparing healthy low- and high-risk men (63–65,134,140,144); and/or 4) increased androgen receptor transactivation activity related to short CAG repeats in the promoter region of the androgen receptor gene (163,182–184). However, there are contradictory data for each of these hypotheses, as indicated earlier and documented in Tables 1–5, and the observed associations were at best weak.

Circulating Androgens and Estrogens

Two of these four hypotheses implicate higher bioavailable circulating androgen levels in high-risk men compared with low-risk populations (191), which may be related to increased androgen production or exposure (136). For example, although body mass index or obesity does not appear to be a risk factor, there are some indications that muscle mass is positively associated with risk, perhaps reflecting exposure to endogenous androgens or anabolic steroids. However, studies of polymorphisms in the CYP17 gene (which encodes for the cytochrome P450C17 α -hydroxylase and 17,20-lyase activity involved in androgen biosynthesis) do not support the notion of a relation between risk and androgen production rates. As will be detailed later, the results of several animal model studies strongly support this contention, but more research is needed to confirm and further define this association in humans and to establish its underlying biologic mechanisms (increased androgen production or 5 α -reductase activity and decreased DHT catabolism) (191). Furthermore, elevated androgen levels do not universally occur in all high-risk groups. Meikle and colleagues (203,204) studied brothers (ages 47–75 years) and sons (ages 22–43 years) of prostate cancer patients who have a threefold to fourfold excess risk compared with unrelated control subjects of the same age ranges. They reported that serum levels of testosterone and DHT were significantly lower, rather than higher as one might expect, in these blood relatives of prostate cancer patients. Because circulating testosterone levels may thus be lower in men with a family history of prostate cancer than in other men, hormonal involvement in familial aggregation of prostate cancer risk seems paradoxical and the involvement of androgens in hereditary prostate cancer may be different from that in sporadic prostate cancer. Zumoff et al. (205) observed that circulating levels of testosterone, but not DHT, were markedly lower in prostate cancer patients younger than 65 years than in those patients 65 years and older. However, control subjects had tes-

tosterone levels that were similar to those of prostate cancer patients of 65 years and older. In several of the studies, summarized in Tables 1 and 2, findings were markedly different when comparing younger (18 to 25–40 years) and older healthy men (>40 years) or comparing younger (<62–70 years) and older men (>62–70 years) with prostate cancer and their age-matched controls. Circulating testosterone levels are also known to paradoxically decrease with aging, whereas prostate cancer risk increases (144–146,206). At the same time, SHBG levels increase with age and estrogen levels remain constant or increase (144,146,206). Thus, bioavailable estrogens and particularly testosterone decrease with increasing age and increasing risk for prostate cancer. This situation may explain the lower prostatic concentrations of DHT with aging reported by Krieg et al. (202) but is in contrast to increasing prostatic estrogen levels they observed with aging. These observations suggest that the role of androgens and estrogens in prostate carcinogenesis may differ in younger men (early onset prostate cancer) and in older men (late-onset cancer) and may be different in men that are at high risk because of familial predisposition and those at high risk associated with their ethnic background or living environment. It is also possible that risk-increasing effects of elevated circulating levels of androgens and estrogens may be effectuated early in life (134,135,141,143) or even before birth (142,207), rather than in the one or two decades preceding the diagnosis of prostate cancer.

Another risk factor may be increased androgen receptor activity related to genetic polymorphisms in the androgen receptor gene. Although body mass index or obesity does not appear to be a risk factor, there are some indications that muscle mass is positively correlated with risk, perhaps reflecting exposure to endogenous androgens or anabolic steroids. Heavy alcohol use accompanied with liver disease may increase risk and be related with decreased clearance of estrogens and elevated circulating estrogen levels. Estrogen levels were also elevated in healthy black men living in the United States compared with European-American men, and this is perhaps associated with the very high risk for prostate cancer of black men living in the United States. However, no association between risk and circulating estrogen levels was found in nested case-control studies (in predominantly European-American cohorts). Thus, the epidemiologic evidence for involvement of androgenic and estrogenic steroid hormones in human prostate carcinogenesis remains inconclusive (191).

Interactions of Environmental, Hormonal, and Racial/Ethnic Factors

The strongest single risk factor for prostate cancer appears to be a western lifestyle, particularly western dietary habits, including a high-fat intake. It is conceivable that dietary risk factors, such as fat, exert their enhancing effects mediated by a hormonal mechanism that involves androgens (11,15,208). For example, heavy alcohol use accompanied with liver disease may increase risk and be related with decreased clearance of estrogens and elevated circulating estrogen levels. However, it is unlikely that lifestyle is the sole factor that explains the differences in prostate cancer risk between Asian and American populations (10,11,136). Genetic factors are probably also important determinants of racial/ethnic disparity of sporadic prostate cancer, such as 5 α -reductase activity or increased androgen receptor

activity related to polymorphisms in the 5 α -reductase or androgen receptor genes.

The single most important combination of risk factors is to be of sub-Saharan African descent and to reside in the United States—African-Americans, as a group, have twice the risk of European-Americans. The reasons for the black-white disparity in prostate cancer rates in the United States are not understood. Environmental exposures (in the broadest sense of the term) are probably responsible for a large fraction of this disparity (10,11,15,209). A relation with similar racial disparities in exposure to potential carcinogens and high-risk dietary habits has been proposed (11,22,59). However, genetic factors, such as polymorphisms in the 5 α -reductase or androgen receptor genes may be related with increased 5 α -reductase activity or androgen receptor activity. However, environmentally influenced hormonal mechanisms may be involved as well, possibly acting *in utero* (207). For example, young, African-American men and pregnant, African-American women have been reported to have higher circulating levels of androgens and estrogens than European-Americans (142). Estrogen levels were also elevated in healthy black men living in the United States compared with European-American men, and this finding is perhaps associated with the very high risk for prostate cancer of black men living in the United States. However, no association between risk and circulating estrogen levels was found in nested case-control studies (in predominantly European-American cohorts).

Conclusions

The epidemiologic evidence for involvement of androgenic and estrogenic steroid hormones in human prostate carcinogenesis remains inconclusive (191). The most promising hormonal risk factor candidates are elevated circulating testosterone and estrogen levels and polymorphisms in the androgen receptor gene associated with increased receptor transactivation activity. In addition, hormonal effects of dietary factors, such as fat, may play a critical role in prostate carcinogenesis in humans, as well as, perhaps, still unexplored/unknown polymorphisms in genes encoding for proteins involved in steroid hormone metabolism and hormone action.

PROSTATE CANCER AND STEROID HORMONES IN LABORATORY ANIMALS

Spontaneously occurring prostate tumors are rare in most species (5–7,210), with exception of the dog and, particularly, humans. It is not understood why prostate cancer is so common in men, whereas it is very rare in almost all other species. There are compelling reasons to implicate hormones, particularly androgenic and estrogenic steroids, in human prostate carcinogenesis, as indicated earlier. The same steroid hormones are also very powerful factors in the induction of prostate cancer in rodent species in which spontaneous prostate neoplasms are rare (15,56,211,212). Pertinent studies concerning the role of androgens and estrogens in experimental prostate carcinogenesis are summarized in the following sections.

It is important to first point out that the various lobes of the rat prostate differ in their propensity to develop prostate carcinomas, either spontaneously or induced by carcinogens or hormones (15,210,211). The rodent prostate, unlike the human or canine prostate, consists of distinct paired lobes: the ventral, dorsal, lateral, and anterior lobes; the dorsal and lateral lobes are

frequently referred to as the dorsolateral prostate, and the anterior lobe is more commonly termed the coagulating gland. In the human and canine prostate, these lobes have merged into one gland, in which different zones have been defined (213). A homologue of the rodent ventral lobe is not present in the human gland (214).

Hormonal Induction

Testosterone

Long-term administration of testosterone induces a low to moderate (5%–56%) incidence of prostate cancer in several rat strains (210,215–220) but not in all strains (221). The induced tumors were adenocarcinomas in all studies but one, in which some squamous cell carcinomas were also observed (218). These carcinomas appeared to develop from the dorsolateral prostate and/or coagulating gland but not from the ventral prostate lobe (210,215–219). The prostate carcinoma incidence in most of these studies was low (5%–20%) (215,218–220). Only the studies reported by Pollard et al. (216,222–225) with the use of the Lobund–Wistar strain sometimes had higher carcinoma incidences, but the incidences varied considerably (0%–60%). In the only other studies with the Lobund–Wistar strain, a low incidence of prostate cancer was found but a high incidence of seminal vesicle tumors was found (219,226). The actual dose of testosterone considerably fluctuated over time in many of these studies from five to 10 times control values down to control values (221,226), but, even when the level of circulating testosterone was kept steadily elevated by twofold to threefold, prostate carcinomas were induced (220). These data indicate that testosterone acts as a complete carcinogen for the rat prostate.

Estrogens and Testosterone

Noble (215) was the first to demonstrate that testosterone is carcinogenic for the rat prostate. He also established that sequential treatment with testosterone and estrogens was even more effective than testosterone per se in the Noble (or NBL) rat strain that he developed. Long-term treatment of NBL rats with a combination of testosterone and estradiol leads to a 100% incidence of adenocarcinomas, which develop from the periurethral ducts of the dorsolateral and anterior prostate (227–229). The development of these tumors is preceded by the appearance of epithelial dysplasia in these ducts and in the acini of the dorsolateral prostate in 100% of treated animals (228–230). Carcinomas developing from the acinar dysplasia in the periphery of the prostate gland have not been observed, but the absence of malignant progression of these lesions, which are morphologically similar to human prostatic intraepithelial neoplasia, has not been established with certainty (228,229). When diethylstilbestrol (DES) was combined with testosterone, the treatment resulted in widespread dysplasia in the ventral prostate, but less or no dysplasia in the dorsolateral prostate (230). Long-term treatment of NBL rats with DES and testosterone induced a low carcinoma incidence in the dorsolateral prostate and some early-stage carcinomas (carcinoma *in situ*) in the ventral lobe (229). When the combined testosterone and estradiol treatment was given to Sprague–Dawley rats, dysplasia developed in the same high frequency as in NBL rats, but the incidence of carcinomas was considerably lower (228,229). Thus, a very high incidence of prostate cancer results from the addition of estrogen to tes-

tosterone treatment, which by itself produces prostate cancer in 35%–40% of treated NBL rats.

Perinatal Estrogen Exposure

Carcinogenic effects of perinatal exposure to DES on the accessory sex glands in male experimental animals have been described in mice, rats, and hamsters (15,231–233). McLachlan and colleagues (231,234) found that 25% of the male offspring of CD-1 mice that had been treated with DES on days 9–16 of gestation had nodular enlargements of the coagulating gland, ampullary glands, and colliculus seminalis at an age of 9–10 months. In one animal, a lesion was found in the area of the coagulating gland and colliculus seminalis that resembled early neoplasia (234). Of eight prenatally DES-exposed male mice that survived for 20–26 months, one had an adenocarcinoma of the coagulating gland, three had hyperplasia of the coagulating gland, two had hyperplasia of the ventral prostate, one had a carcinoma of the seminal vesicle, and two had squamous metaplasia of the seminal vesicle (231,232). No such lesions occurred in control animals. Prenatal DES exposure of mice also induces testicular tumors (particularly of the rete testis) and non-neoplastic lesions in the testes and epididymis (235).

Treatment of Han:NMRI mice with DES or estradiol on the first 3 days of life resulted in a 90%–100% incidence of epithelial dysplasia of the periurethral glands and of the periurethral proximal parts of the dorsolateral prostate, coagulating glands, and seminal vesicles after 12–18 months (236,237). Subsequent treatment with DHT and estradiol from 9–12 months of age increased the severity of the dysplasia when the prostates were examined at 12 months, suggesting permanent estrogen hypersensitivity of these tissues. Arai et al. (232) treated Wistar rats with DES for the first 30 days of life. One group was neonatally castrated and the second group remained intact. Two of 11 castrated, DES-exposed rats developed squamous cell carcinomas in the area of the dorsolateral prostate, coagulating gland, and ejaculatory ducts, and all these animals had papillary hyperplasia and squamous metaplasia of the coagulating gland and ejaculatory duct. Squamous metaplasia was also found in some of eight noncastrated DES-exposed rats, but no hyperplasia or neoplasia developed. Vorherr et al. (238) obtained similar results in rats exposed prenatally and/or neonatally to DES.

The results of these studies demonstrate that prenatal and neonatal estrogen exposure of rodents can be carcinogenic for the prostate. Data also suggest that these treatments may imprint permanent alterations in the hormonal sensitivity of the prostate that may play a role in the carcinogenic effect of perinatal estrogen exposure.

Induction by Chemical Carcinogens and Hormones

Exposure to Chemical Carcinogens Combined With Hormonal Stimulation of Cell Proliferation

Very few reports are available of induction of prostate tumors by chemical carcinogens administered systemically or via the oral or inhalation routes. Only two organic chemical carcinogens induce prostate adenocarcinomas on systemic administration, without any additional concomitant or subsequent treatment, *N*-nitrosobis(oxopropyl)amine (BOP) and 3,2'-dimethyl-4-aminobiphenyl (DMAB) (239,240). Direct application of chemical carcinogens to prostate tissue in experimental animals produces sarcomas or squamous cell carcinomas (7,241).

Short-term hormonal stimulation of cell proliferation in the prostate at the time of carcinogen administration has been demonstrated to increase the sensitivity of the target cells for tumor induction. Dorsolateral prostate adenocarcinomas have been produced at 5%–25% incidence when prostatic cell proliferation was stimulated in combination with treatment with indirect-acting carcinogens (such as DMAB and 7,12-dimethylbenz[*a*]anthracene) and direct-acting chemical carcinogens (such as *N*-methyl-*N*-nitrosourea [MNU]); these carcinogens do not induce these tumors when administered alone (220,242–245). However, in some studies, only a very small or no enhancing effect was found of stimulation of prostatic cell proliferation on prostate carcinoma induction by carcinogens (218, 221,246,247). Nevertheless, stimulation of cell proliferation appears to be co-carcinogenic for prostate cancer induction by many chemical carcinogens.

Testosterone as Tumor Promoter of Prostate Carcinogenesis

Long-term administration of testosterone to rats markedly enhances prostatic carcinogenesis following initial treatment with chemical carcinogens that target the prostate because of tissue-specific metabolism (DMAB and BOP) and/or concurrent hormonal stimulation of prostatic cell proliferation (70,71,211, 217–221,223,225,248). This enhancement may not occur if certain requirements are not adequately met (210,211,218). For example, after a single injection of BOP or MNU given to F344 rats without concurrent stimulation of prostatic cell proliferation, long-term testosterone treatment did not enhance prostatic carcinogenesis (221). High incidences (66%–83%) of adenocarcinomas of the dorsolateral and/or anterior prostate, but not the ventral prostate, were induced by chronic treatment with testosterone, following a single administration of MNU or BOP given during stimulation of prostatic cell proliferation in Wistar rats, or during and after 10 repeated biweekly injections of DMAB in F344 rats (70,71,210,211,218,220,221,248). This effect is somewhat strain dependent, because when the same treatments were given to Lobund–Wistar rats, rather variable prostate carcinoma incidences of between 50% and 97% were reported by Pollard and colleagues (217,223,225) and only a 21%–24% incidence was found by Hoover et al. (219) and Tamano et al. (226).

The enhancing effect of testosterone on prostate carcinogenesis is remarkably confined to the dorsolateral and anterior prostate, and no tumors occur in the ventral prostate. In fact, long-term testosterone treatment produces a shift of the site of DMAB- and BOP-induced carcinoma occurrence from exclusively the ventral lobe to predominantly the dorsolateral and anterior lobes (218,221,248). The dose–response relationship between testosterone dose and prostate carcinoma yield is very steep. A slight (less than 1.5-fold) elevation of circulating testosterone levels is sufficient for a near-maximal enhancement of the tumor response, and a twofold to threefold elevation is sufficient for a maximal response. These concentrations are within the normal range of circulating testosterone levels in the rat (220). Thus, testosterone is a powerful tumor promoter for the rat prostate.

Effects of Testosterone on Prostate Cancer Induction by Cadmium and Ionizing Radiation

Cadmium can be carcinogenic for the rat ventral prostate as demonstrated by Waalkes et al. (249,250). The selective sensi-

tivity of the ventral prostate lobe for the carcinogenic action of cadmium is most likely due to its lack of cadmium-binding proteins (251). A single injection of cadmium chloride produced *in situ* (noninvasive) carcinomas in the ventral lobe provided that cadmium-induced testicular toxicity was avoided, either by keeping the cadmium dose below 5 mg/kg, by intramuscular rather than subcutaneous administration of the cadmium, or by antagonizing the testicular toxicity of cadmium by simultaneous administration of sufficient amounts of zinc. These observations indicate that cadmium induces proliferative lesions in the rat ventral prostate only when testicular function, conceivably testosterone production, is intact. In addition, these data suggest that androgens also act as tumor promoters in this system, but this hypothesis has not been tested. Other mechanisms may also be involved, because, for example, testosterone considerably increases cadmium disposition and retention in the rat ventral prostate (252).

Local x-ray exposure of the pelvis has been shown to induce prostate carcinomas in ICR/JCL mice (253) and in Sprague–Dawley rats (254). Prostate carcinomas (33% incidence) developed only in rats that were castrated and received androgen replacement prior to irradiation, but intact and only castrated rats did not develop prostate cancer following irradiation. These observations suggest that testosterone treatment was required for tumor development, perhaps as tumor promoter (254).

Prostate Cancer and Steroid Hormones in Laboratory Animals: Summary and Conclusions

Stimulation of prostatic epithelial cell proliferation by androgens during exposure to chemical carcinogens increases the susceptibility of the rat prostate to cancer induction in a co-carcinogenic fashion. Testosterone appears to be a weak complete carcinogen, but it is a very strong tumor promoter for the rat prostate at near-physiologic plasma concentrations (220). The very powerful tumor-promoting activity of androgens perhaps explains their weak complete carcinogenic activity on the rat prostate. A slight elevation of circulating testosterone can lead to a marked increase in prostate cancer in rat models. This observation is highly relevant in view of the aforementioned possible weak association between human prostate cancer risk and slightly elevated circulating androgen levels found in some epidemiologic studies (191). Thus, the experimental data provide strong support for the concept that minimal increases in circulating androgens may have substantial enhancing effects on prostate cancer risk. The addition of estradiol to chronic treatment with testosterone strongly enhances the carcinogenic activity of the androgen for the rat dorsolateral prostate. The sensitivity for the carcinogenic effects of this hormone combination appears to be confined to the periurethral, proximal ducts of the dorsolateral and anterior prostate. The estradiol plus testosterone treatment also induces acinar lesions that are similar to human prostatic intraepithelial neoplasia. These observations strongly suggest a critical role for estrogens in prostate carcinogenesis. Perinatal estrogen exposure is also carcinogenic for the male rodent accessory sex glands. The periurethral, proximal ducts of the dorsolateral and anterior prostate and seminal vesicle and the intraprostatic urethral epithelium appear to be the most sensitive rodent male genital tract tissue to the carcinogenic effects of perinatal estrogen exposure. Of interest in this regard is the report by Driscoll and Taylor (255) of hypertrophy and squamous metaplasia of the prostatic utricle and prostatic ducts in

55%–71% of 31 infants that had been exposed to DES *in utero* and had died perinatally from unrelated causes. Such squamous metaplastic changes have also been reported to occur in human fetal prostatic tissue transplanted into nude mice that were subsequently treated with DES (256). These human observations suggest that the DES findings in rodents may have human relevance.

MECHANISMS OF HORMONAL PROSTATE CARCINOGENESIS

As stipulated before, there are compelling reasons to assume that androgens play a critical role in prostate carcinogenesis, and there is experimental evidence to suggest that estrogens are involved as well (56). Because of the hormonal nature of these steroids, receptor mediation has been proposed as the major mechanism by which androgens and estrogens act in the causation of prostate cancer (257). For androgens, mechanisms other than those mediated by androgen receptors seem unlikely, except for the generation of estrogens via aromatization. For estrogens, however, nonreceptor-mediated genotoxic effects are conceivable, in addition to receptor-mediated processes (56). These various potential mechanisms are discussed in the following sections.

Stromal–Epithelial Interactions

First, it is important to emphasize that considerable evidence indicates interactions between epithelial and stromal cells in the normal prostate. Such interactions are undoubtedly critical and may be essential in prostate carcinogenesis as well, because prostatic mesenchyme is known to be a mediator of androgen action in the developing and adult rodent prostate and possibly the human prostate (258,259). No studies, however, have directly addressed the role of stromal–epithelial interaction in human or rodent prostate carcinogenesis. Krieg et al. (202) studied steroid hormone concentrations in stromal and epithelial compartments of normal human prostates from subjects varying from 20 to 80 years of age. DHT concentrations in the epithelium decreased considerably with aging, but they remained stable in stromal cells, whereas testosterone concentrations appeared unaffected by age in either compartment. These data suggest that the activity of 5 α -reductase in the epithelium decreases with aging but remains intact in the stroma. However, concentrations of estradiol and estrone in the stroma, but not the epithelium, increased markedly with aging. These observations suggest that the prostatic stroma is an important site for both androgen and estrogen action and metabolism, such as aromatase activity, which seems to increase with aging because estrogens accumulate with aging and androgen levels remain stable. This is unlike the concentrations of estrogens and androgens in the circulation or in epithelial cells, where both decrease. Thus, it is conceivable that with aging and increasing risk for prostate cancer the prostatic stroma continues to be an important androgen signal mediator to the epithelium and is an increasingly important local producer of estrogens.

Role of Androgens in Prostate Carcinogenesis

The results of the earlier summarized rodent experiments clearly indicate carcinogenic and strong tumor-promoting properties of androgens, and the results of a limited number of epidemiologic studies provide some support for the notion that androgens may have such effects in humans. However, the

mechanisms of the carcinogenic and tumor-promoting effects of androgens on the rodent prostate are not known with certainty. The very steep relationship between testosterone dose and prostate carcinoma response in rat models is suggestive of involvement of an androgen receptor-mediated mechanism (220). Other mechanisms may nevertheless be involved as well. For example, Ripple et al. (260) observed increases in indicators of oxidative stress in androgen-sensitive LNCaP human prostate cancer cells exposed to DHT, although it is possible that these effects were androgen receptor mediated.

Stimulation of Cell Proliferation and Carcinogenic and Tumor-Promoting Effects of Androgens

The postulated role of androgens in human prostate carcinogenesis has been ascribed to their androgen receptor-mediated stimulating effects on prostatic cell proliferation (136,257). No direct evidence, however, is available that elevation of circulating testosterone leads to increased cell proliferation in the human prostate. It has been well established that androgen administration to castrated rodents causes elevation of prostatic cell proliferation similar to that observed in cell cycle synchronization experiments with cells *in vitro*. However, the increase in prostatic cell proliferation caused by testosterone or DHT administration in castrated rodents is only transient, and after a few days cell turnover returns to its normal very low levels (261). Thus, continued androgen treatment of rodents does not result in permanently elevated cell proliferation rates in the male accessory sex glands, but rather appears to support differentiation. Furthermore, DHT may even suppress prostatic cell proliferation in intact rats (228). Thus, a mere continuous stimulation of cell proliferation is unlikely to be the major mechanism of the enhancing effects of testosterone on prostatic cancer induction in rodents and possibly humans.

Conceivably other, nonhormonal factors affect prostatic cell proliferation. For example, over the lifetime of a man, the prostate undergoes repeated inflammatory insults (prostatitis) with reactive cell proliferation and generation of reactive oxygen species as possible consequences (262,263), and sexual activity conceivably also affects prostatic cell turnover. Support for a cell proliferation hypothesis is provided by rodent experiments that indicate that increased prostatic cell proliferation at the time of exposure to carcinogens can enhance the sensitivity of the tissue to the carcinogenic effects of these agents (220,242–245). Stimulation of cell proliferation during carcinogen exposure increases the likelihood that promutagenic DNA damage, such as carcinogen–DNA adducts, will be fixed as permanent mutations. In humans, increased cell proliferation may thus enhance the carcinogenic effects of low-level exposure to environmental and endogenous carcinogens.

The rate of cell proliferation at the time of carcinogen exposure may be only one of several androgen-related factors that determine sensitivity of the prostate to cancer induction by carcinogens through androgen-receptor mediated mechanisms. For example, Sukumar et al. (264) have hypothesized that prostate cells that harbor critical genetic alterations, such as activating point mutations in oncogenes or inactivating alterations in tumor suppressor genes, may be selectively sensitive to induction of the cell proliferation, rather than cellular differentiation, by androgens. However, this hypothesis has not been critically tested. These cells could thus have a selective growth advantage over normal cells, which do not respond to chronic

testosterone treatment with sustained proliferation (221). It is also possible that androgens, in addition to other factors, influence the effectiveness of indirect-acting carcinogens that are metabolically activated and otherwise metabolized in the prostate itself.

Role of Androgen Metabolism and Androgen Receptor Sensitivity

Pertinent to any hypothesis implicating androgens in prostate carcinogenesis are considerations related to androgen receptor function and androgen metabolism, from steroid biosynthesis, to conversion of testosterone to DHT and to DHT catabolism. Ross et al. (136) have developed the idea that genetically determined differences in the activities of steroid biosynthetic enzymes, 5 α -reductase, and enzymes that metabolize DHT, as well as in androgen receptor activity are major determinants of risk both at the population and at individual levels [see also (18)]. Functional polymorphisms in the genes that encode for these enzymes and the androgen receptor have been hypothesized to underlie this notion (18,136).

The evidence for these polymorphisms being important in human prostate carcinogenesis has been summarized in detail and evaluated together with the results of endocrinologic studies in earlier sections. The overall conclusions were that to date there is inconsistent and conflicting evidence that functional polymorphisms in the 5 α -reductase gene and differences in 5 α -reductase activity are important determinants of prostate cancer risk. However, there is stronger evidence to suggest that risk is associated with a functional polymorphism in the androgen receptor gene, short lengths of CAG repeats in the transactivation domain of the protein that are linked with increased transactivation activity *in vitro* (137–139,163,164,182–185). However, this association is weak at best. Several other polymorphisms identified in genes encoding for the androgen receptor and other androgen metabolizing enzymes studied have been unevenly distributed among populations that differ in prostate cancer risk (152–156,160,162) or to be associated with risk in case-control studies (153–155,163,170,182). These studies concerned polymorphisms with unknown functional significance in genes encoding the type II 3 β -hydroxysteroid dehydrogenase that catabolizes DHT, the cytochrome P450C17 α enzyme that has 17 α -hydroxylase and 17,20-lyase activity involved in androgen biosynthesis, and CCG or GGN repeats in the promoter region of the androgen receptor gene. The study of these types of polymorphisms is a rapidly evolving field of investigation and will no doubt lead to significant and relevant new findings in the near future (136).

The observation of slightly, but mostly not significantly, higher circulating testosterone levels in high-risk African-American men compared with lower-risk European men suggests that their rates of androgen biosynthesis may be higher. Although lower as well as higher testosterone concentrations have been found in lower-risk Asian or African men compared with European- or African-Americans, testosterone levels were lower in Asians living in Asia than in American populations regardless of their ethnicity in the only two studies that included Asian populations. In addition, directly measured testosterone production rates were lower in Chinese in China than in both Chinese-Americans and European-Americans (148). These observations are consistent with the hypothesis that environmental factors, such as diet, determine prostate cancer risk at the

population level by influencing androgen production such that they are lower in low-risk than in high-risk circumstances (208).

Assessing the role of androgen in prostate carcinogenesis is complicated by the fact that the prostatic stroma is an important site for androgen action and metabolism in the prostate in addition to the epithelium. For example, epithelial DHT concentrations decline dramatically with aging, but they remain stable in the stroma even though the source for intraprostatic DHT, circulating testosterone, also diminishes with aging. This observation suggests that stromal 5 α -reductase activity remains stable, whereas epithelial activity of this enzyme declines with aging. These observations illustrate the difficulties in interpreting the results of studies of circulating androgenic (or other) hormone levels of genomic polymorphisms in relevant genes, because they do not necessarily provide relevant information about what is going on at the level of the prostatic epithelial cell and its important immediate environment, the prostatic stroma.

Role of Estrogen in Prostate Carcinogenesis

The results of the earlier summarized epidemiologic studies provide limited evidence for an association between prostate cancer risk and circulating levels of estrogens, which appear to be higher in men of African descent younger than 50 years of age than in European-American men. This observation suggests that estrogens may be involved in prostate carcinogenesis, because men of African descent living in an American environment have the highest risk for prostate cancer of any population.

Most of the direct evidence in support for a role of estrogens in prostate carcinogenesis comes from studies with treatment of NBL rats with testosterone and estradiol (229,265). It appears that the estrogen-related mechanisms underlying this effect are a mixture of estrogen receptor-mediated and nonreceptor processes, which are discussed in the following paragraphs. In addition, there is evidence to suggest that the mechanisms involved in hormonal induction of rat prostate cancer, originating from periurethral prostatic ducts, are different from those involved in the induction by testosterone and estradiol of dysplastic lesions developing in the dorsolateral prostate acini.

As alluded to earlier, there is evidence for the presence of the CYP19 enzyme aromatase in the human prostate, which could provide a local source of estrogens from conversion of testosterone (195–199), but there are contradictory reports (200,201). The local production of estrogens in the human prostate is possibly a stromal process, and stromal aromatase activity may increase with aging (202). Data on the presence of aromatase in the rodent prostate are also somewhat contradictory, because aromatase activity has been reported in the rat ventral prostate and a transplantable rat prostate carcinoma (266), but it was not detectable in mouse prostate (267). These discrepancies, which may be due to interspecies or methodologic differences, point to the need for further research.

Estrogen Receptor-Mediated Mechanisms

Estrogen receptors are found in the prostate, and Lau et al. (268) demonstrated that both the estrogen receptor- α and - β are present in the rat prostate. Thus, direct receptor-mediated effects of estrogens on the prostate are plausible. However, rodent studies that used antiestrogen treatments (tamoxifen and ICI-182,780) have yielded contradictory results about the involve-

ment of estrogen receptor mechanisms in prostate carcinogenesis. The prostate tumor-promoting effects of testosterone may involve estrogen generated by aromatization. However, simultaneous administration of testosterone and tamoxifen failed to alter the prostate carcinogenesis-enhancing effect of the androgen in an experiment in rats injected with DMAB prior to the hormone treatment (269). However, ICI-182,780 blocked the induction of epithelial dysplasia in the prostatic periphery in NBL rats treated with testosterone and estradiol for 16 weeks (270); the effects of this antiestrogen on induction of periurethral prostate carcinomas are not known.

Leav et al. (228) and Ofner et al. (230) showed that dorsolateral prostatic tissue with epithelial dysplasia from NBL rats treated with testosterone and estradiol for 16 weeks accumulates estradiol and 5α -androstane- 3β , 17β -diol, a weak estrogenic agonist; this accumulation of estrogenic species does not occur in the ventral lobe, which also does not develop dysplasia. In rats treated with testosterone and DES for 16 weeks, dysplasia developed more distinctly in the ventral than in the dorsolateral prostate, as indicated earlier. This development coincided with a preferential accumulation of estradiol and 5α -androstane- 3β , 17β -diol in the ventral prostate (230). These observations suggest that increased levels of estradiol and the weakly estrogenic androgen metabolite in prostatic target tissue may be causally related with the development of hormone-induced dysplasia and perhaps carcinomas in the NBL rat model (230).

In tissue with epithelial dysplasia from the dorsolateral prostatic periphery that was derived from rats treated with testosterone and estradiol for 16 weeks, elevated levels of nuclear, but not cytosolic, type II (intermediate-affinity) estrogen-binding sites, but not type I (high-affinity) binding sites, have been found (228,271). The type II estrogen receptor is a cell proliferation marker believed to be a key factor in normal and aberrant growth regulation in female estrogen target tissues. These data indicate that protracted stimulation of cell proliferation may be involved in the formation of hormone-induced rat prostate dysplasia (228,271). Indeed, mitotic indices in testosterone plus estrogen-treated NBL rat dorsolateral prostate were increased over control values; this increased mitotic activity was largely confined to the dysplastic lesions (228,271).

One well-established effect of estrogen treatment in rodents is stimulation of prolactin secretion. This finding raises the possibility that some or even all estrogen effects on the rodent prostate may be mediated through elevation of prolactin secretion, and there is some experimental support for this notion. Transplantation of a prolactin-producing pituitary tumor into rats treated with DMAB enhanced the formation of atypical hyperplasia, a preneoplastic lesion, but not carcinomas, in the ventral prostate, and treatment with bromocriptine, a prolactin secretion-suppressing agent, counteracted this effect (272). Bromocriptine also lowered the formation of ventral prostatic atypical hyperplasia and carcinomas in rats treated with only DMAB. Development of epithelial dysplasia in the dorsolateral prostatic periphery of NBL rats treated with testosterone and estradiol for 16 weeks was also blocked by bromocriptine, but effects on periurethral carcinoma development were not studied (273). Thus, there is evidence to suggest that prolactin may modulate the induction of preneoplastic lesions in the rat prostate, but the relevance of these findings for prostate cancer development are not clear.

In conclusion, several lines of evidence are available to sug-

gest that estrogen receptor-mediated mechanisms contribute to the induction of prostate cancer by hormonal treatments, but conclusive data in this regard are largely lacking.

Nonreceptor Mechanisms

Estrogens have been shown to be capable of producing DNA damage in target tissues susceptible to estrogen-induced carcinogenesis, independent of their interaction with the estrogen receptor, as discussed in detail elsewhere in this monograph. In the kidney of male hamsters treated with DES, Liehr and colleagues (275) have found a direct DES-DNA adduct and indirect estrogen-generated DNA adducts perhaps of endogenous origin and of undetermined structure detectable by ^{32}P -postlabeling (276). Both observations are thought to be related to the formation of catechol estrogens that undergo redox cycling during which reactive intermediates and reactive oxygen species are generated and lipid peroxidation can be initiated (274). Similar observations have been made in the prostate of NBL rats treated for 16 weeks with testosterone plus estradiol. This treatment enhanced the formation of a chromatographically unique endogenous adduct selectively in the periurethral region of the rat dorsolateral prostate, which is the site of the carcinogenic effect of this treatment [(277) Bosland et al., unpublished data; *see also* Chapter 4]. Ho and Roy (278) reported increased single-strand DNA breaks and accumulation of fluorescent lipid peroxidation products in the dorsolateral prostate of NBL rats after this treatment, but they did not separately analyze the periurethral and peripheral areas of the prostate. In addition, substantially elevated levels of 8-hydroxydeoxyguanosine and, to a lesser extent, lipid hydroperoxides have been found at the periurethral tissue but not in the peripheral area of these glands (Bosland et al., unpublished data; *see also* Chapter 4). Lower, but still elevated, levels of the endogenous DNA adduct detectable by ^{32}P -postlabeling, 8-hydroxydeoxyguanosine, and lipid hydroperoxides were also found in the periurethral prostate of rats treated with only testosterone, perhaps due to formation of estrogens by aromatization (Bosland et al., unpublished data). The enhancement of endogenous DNA adduct formation, oxidative DNA damage, and lipid peroxidation selectively at the site of tumor formation and preceding it strongly suggests that these effects are causally involved in the carcinogenic effect of the hormone treatment. It is likely, but as yet unproven, that the exogenously administered estrogens or formation of estrogen via aromatization of testosterone and a genotoxic mechanism are critical to the carcinogenic effect of this hormone combination for the prostate, rather than other mechanisms, including receptor mediation.

This hypothesis implies that catechol estrogen formation occurs at the relevant site within the prostate, as indicated earlier and discussed in detail elsewhere in this monograph. Lane et al. (212) demonstrated that microsomes isolated from testosterone plus estradiol-treated NBL rat dorsolateral prostate do not appear to be able to generate the catechol estrogens 2-hydroxy and 4-hydroxy-estradiol and -estrone. However, because periurethral prostate tissue was not incorporated in the analysis and the relevance of such microsomal assays for the *in vivo* situation is unclear, these data do not refute the possibility of catechol estrogen formation in the periurethral prostate. Furthermore, it is conceivable that the mechanisms of induction of dysplasia in the dorsolateral glandular prostate (estrogen receptor-mediated, possibly not involving estrogen-generated genotoxic processes) are

different from those involved in generation of the periurethral prostatic carcinomas (estrogen-generated genotoxicity, but possibly no estrogen receptor mediation). This idea leads to the hypothesis that 1) testosterone acts as a tumor promoter and estrogens act as genotoxic "tumor initiators" in the testosterone plus estradiol-treated NBL rat model of (periurethral) prostate carcinogenesis, and 2) the androgen also acts as enhancer of induction of dysplasia (periphery) in this model, which requires conjunct action of estrogen via estrogen receptors. However, these hypotheses remain to be critically tested.

The human relevance of these findings in the testosterone plus estradiol-treated NBL rat model remains unclear at present. However, oxidative DNA damage and lipid peroxidation reflective of reactive oxygen damage have been observed in the human prostate (279), and signs of increased oxidative stress have been found in patients with prostate cancer as compared with control subjects (280). Whether these observations are estrogen-exposure related or associated with other risk factors, such as a high fat diet (263), is not known, but they suggest that endogenous oxidative stress may be important in human prostate carcinogenesis and are consistent with involvement of estrogen-generated oxidative DNA damage.

Perinatal Estrogen Exposure: Imprinting

As summarized earlier, perinatal estrogen exposure of mice resulted in epithelial dysplasia of the periurethral proximal parts of the dorsolateral and anterior prostate and of the seminal vesicles (236,237) as well as carcinomas in these areas (234). In addition, mice that were neonatally estrogenized hyperresponded to secondary estrogen treatment (estradiol) with the development of considerable squamous metaplasia in these same tissues, but control subjects responded with little or no squamous change (236). These very same tissue areas possess estrogen receptors, which indicate their estrogen sensitivity (236). However, the activity of estradiol hydroxysteroid oxidoreductase, a marker of estrogen sensitivity, and incorporation of tritiated thymidine in epithelial compartments of these tissues were not changed in response to secondary treatments with estradiol in neonatally estrogenized mice (236,237). In response to secondary androgen treatment (DHT), tritiated thymidine incorporation was markedly increased selectively in stromal cells of the anterior and ventral prostate, indicating a lasting effect of neonatal estrogen exposure on the androgen responsiveness of the stromal component of the mouse prostate (236). These observations suggest that perinatal estrogen exposure of mice imprints lasting alterations in estrogen and androgen responsiveness of the male accessory sex glands.

The exact mechanism of these complex imprinting effects is not clear. Perinatal estrogen treatment may act indirectly on the male accessory sex glands by imprinting permanent alterations in the secretion of pituitary hormones and testicular androgen, or directly by, e.g., imprinting altered expression of androgen, estrogen, and prolactin receptors or changes in steroid metabolism in the accessory sex gland, which all may result in modified development of these glands (281–284). For example, in neonatally estrogenized mice, luteinizing hormone and follicle-stimulating hormone plasma levels were found to be elevated (282), whereas circulating testosterone levels were decreased (281,284) or unaltered (282). However, no abnormalities in circulating estrogen and androgen levels were found in boys that had been exposed to DES *in utero* (285). Prostatic DHT forma-

tion by 5 α -reductase was found to be impaired in adult mice neonatally treated with DES (267). Nuclear androgen receptor levels in these mice were decreased in the dorsal and ventral prostate but not affected in the lateral lobe, and the number of androgen receptor-positive stromal cells was increased in all three lobes (284). The significance of these findings for the carcinogenic effects of perinatal estrogen exposure to mice is not clear. Although the exact mechanisms of the carcinogenic effects of perinatal estrogen exposure for the prostate remain unclear, there appear to be lasting direct and indirect effects of this treatment on the mouse prostate. The human relevance of the findings in mice remains unclear at present, but *in utero* estrogen exposure is likely to occur in humans (142).

OVERALL CONCLUSIONS

With the exception of "exposure" to a western lifestyle, including a high-fat diet, an African-American "environment," and, perhaps, venereal disease and unknown factors related to farming and employment in armed services and nuclear industry, there are no known exogenous exposures that are associated with prostate cancer risk, and none of these circumstances constitute exposures to specific chemicals or factors. Familial aggregation of prostate cancer risk is consistently observed and confers a considerable increase in risk but explains less than 10% of all cases. Putative susceptibility loci have been identified, but there are no indications that these loci are related to hormonal factors. This lack of known specific risk factors is remarkable in view of the high frequency of this malignancy in western countries. It may indicate that there are many exogenous risk factors for prostate cancer that are too ubiquitous and overlapping to be detectable by epidemiologists. However, it is possible that there are strong endogenous determinants of prostate cancer risk that are "overpowering" most exogenous risk factors in epidemiologic analyses.

Androgenic hormones and androgen receptor mechanisms are prime candidates to be such important endogenous factors, but the epidemiologic evidence in favor of this view is weak. Elevation of bioavailable and bioactive androgens in the circulation and in the target tissue as an important risk factor is biologically very plausible. The results of several animal model studies strongly support this contention. Some experiments indicate that substantial enhancement of prostate carcinogenesis can be produced by very small elevations of circulating testosterone, which, if also valid for humans, may explain why the epidemiologic associations between circulating androgen levels and prostate cancer are weak at best. Evidence is also available indicating that increased transactivation activity of the androgen receptor may be associated with increased prostate cancer risk, both at the population and individual levels. However, more research is needed to confirm and further define these associations in humans and to further unravel the biologic mechanisms underlying the increased risk that may be associated with elevated circulating androgen levels and increased androgen receptor sensitivity.

African-American men have a twofold higher risk than European-American men do. The unknown environmental and possibly genetic factors that determine the high prostate cancer risk in African-American men may act through modifying their hormonal status. Indeed, circulating levels of androgens and, in men younger than 50 years, estrogens appear to be higher in men of African descent than in European-American men. Such endo-

crine mechanisms perhaps act as early as *in utero*, because circulating levels of androgens and estrogens have been shown to be slightly higher in young men and in pregnant African-American women than in European-American women.

Hormonal stimulation of prostatic epithelial cell proliferation enhances the susceptibility of the rat prostate to chemical carcinogens. Testosterone at near-physiologic plasma concentrations is a weak complete carcinogen and a strong tumor promoter for the rat prostate. The very strong tumor-promoting activity of androgens possibly explains their weak complete carcinogenic activity. The mechanism of the tumor-inducing and -promoting activities of androgens for the rat prostate is unknown. It is unlikely that chronic stimulation of prostatic cell proliferation rates by androgens is involved. However, it is possible that prostatic epithelial cells that carry critical genetic alterations have a selective growth advantage over normal cells and do not respond to androgens by differentiation, as normal cells would, but by proliferation.

Chronic exposure to testosterone plus estradiol is strongly carcinogenic for the dorsolateral prostate of some rat strains, whereas testosterone alone is only weakly carcinogenic. The mechanism of this carcinogenic effect in the rat prostate is incompletely understood, but it appears to involve estrogen-generated oxidative stress and genotoxicity and also requires androgen- and estrogen receptor-mediated processes, such as changes in sex steroid metabolism and receptor status. There is evidence for the presence of the enzyme aromatase in the human and rat prostate, providing a local source of estrogens, which in humans seem to increase in activity with aging. Perinatal estrogen exposure is carcinogenic for the rodent male accessory sex glands. Hyperplastic and squamous metaplastic changes have been reported in human genital tract tissue following prenatal DES exposure, indicating that prenatal exposure to DES may also target the human prostate. The mechanisms of these prenatal estrogen effects are not clear, but they may involve permanently imprinted changes in hormone production and tissue hormone sensitivity.

From these observations, the following multifactorial general hypothesis of prostate carcinogenesis emerges: Androgens act as strong tumor promoters via androgen receptor-mediated mechanisms to enhance the carcinogenic activity of strong endogenous genotoxic carcinogens, including reactive estrogen metabolites and estrogen- and prostatitis-generated reactive oxygen species, and possibly unknown weak environmental carcinogens. All these processes are modulated by a variety of environmental factors, such as diet, and by genetic determinants, such as hereditary susceptibility genes and polymorphic genes that encode receptors and enzymes involved in the metabolism and action of steroid hormones.

FUTURE RESEARCH NEEDS

This overview clearly indicates that, although steroid hormonal factors are strongly implicated in prostate carcinogenesis, we know very little about their involvement. Considerable research is needed to further our understanding of this relationship. Some promising areas for future research are summarized below. One aspect to be mentioned up front is that the African-American population offers unparalleled but vastly underexploited opportunities for such research, which may also lead to new insights in the possible prevention of prostate cancer in this underrepresented but disproportionately affected group.

- 1) To resolve the uncertainties about the importance of circulating hormone levels, additional, large, nested case-control studies are needed by using cohorts of men belonging to diverse racial/ethnic and other groups that differ substantially in risk of prostate cancer with serial measurements of circulating steroid and other hormones (both over time to assess consistency and trends and within 24-hour periods to assess circadian rhythm variations).
- 2) To address the functional significance of polymorphisms in genes encoding for enzymes involved in steroid hormone biosynthesis and metabolism, studies are needed of correlations between circulating hormone levels and these polymorphisms in relation to prostate cancer risk.
- 3) Even more important than the studies mentioned above, there is an urgent need to develop strategies to examine the relationships of circulating hormone levels and genetic polymorphisms in genes encoding for relevant enzymes with intraprostatic hormone levels, activities of steroid hormone metabolizing enzymes, and androgen receptor mechanisms.
- 4) To determine the importance of estrogen-generated gene damage, studies in humans and animal models are needed of DNA damage and mutations in the prostate associated with exposure to estrogens (and possibly other steroid hormones) in relation to risk of prostate cancer.
- 5) To assess the involvement of gene-environment interactions in prostate carcinogenesis, studies are needed in humans and animal models of the effects of diet and other environmental factors on circulating and prostatic hormone levels, intraprostatic activities of steroid hormone metabolizing enzymes, and prostatic androgen receptor function.
- 6) To expand understanding of the importance of genetic polymorphisms in prostate carcinogenesis, intensification is needed of searches for new relevant polymorphisms in genes encoding for enzymes involved in steroid hormone biosynthesis and metabolism, as well as factors involved in androgen receptor function, including determination of their function.
- 7) Finally, to facilitate many of the research needs listed above, there is the need for establishing banks of adequate DNA, serum, and prostatic tissue samples in large, well-documented, relevant cohorts of aging men.

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Chapter 3: Endogenous Estrogens as Carcinogens Through Metabolic Activation

James D. Yager

A common thread linking the main risks for developing breast cancer in women is cumulative, excessive exposure to estrogen. The standard paradigm to account for this association focuses on increased cell proliferation caused by estrogen through estrogen receptor-mediated signal transduction accompanied by increased probability for mutation to occur during DNA synthesis. This chapter provides an overview of the mounting evidence, provided from cell culture and whole animal experimental studies, in support of a role for the oxidative metabolites of estrogen, in particular, the catechol estrogens, in the development of estrogen carcinogenesis. This provides a paradigm for how estrogens may contribute to the development of human breast cancer. The chapters that follow will fill in the details. Evidence shows that the catechols themselves are signaling molecules that work through the estrogen receptor. In addition, upon further oxidation, the catechols can give rise to reactive quinones capable of forming direct adducts with glutathione and purines in DNA and of redox cycling to generate reactive oxygen species that can cause oxidative damage. Estradiol and estrone, as well as their 4-hydroxy catechols, are carcinogenic in the Syrian golden hamster kidney, and ethinyl estradiol is a strong promoter of hepatocarcinogenesis in the rat. Increased oxidative DNA damage has been detected in target tissues after estrogen treatment in both animal model systems. Furthermore, several recent molecular epidemiologic studies have found that a polymorphism associated with a low-activity form of catechol-*O*-methyltransferase, an enzyme involved in the inactivation of catechol estrogens, is associated with an increased risk for developing breast cancer. The increased risk is observed in certain women, although the studies are not consistent on which subgroup of women (e.g., premenopausal or postmenopausal) is at increased risk, and one study detected no increased risk. Reasons for such discrepancies are discussed in light of factors, such as genetic polymorphisms and environmental/lifestyle susceptibility factors, which control the tissue-specific balance within cells among the estrogen metabolites. It is concluded that such factors will have to be identified through additional mechanistic studies and that, as they are identified, they can be incorporated into future molecular epidemiologic studies designed to determine their actual impact on cancer risk in human populations. [J Natl Cancer Inst Monogr 2000;27:67-73]

For a substantial fraction of breast cancer cases in women, well-established risk factors, revealed by epidemiologic studies, include early age at menarche, late first full-term pregnancy, nulliparity, late menopause, family history of breast cancer, socioeconomic status, and perhaps estrogen replacement therapy (1-5). A common thread linking these factors is cumulative, excessive lifetime exposure to estrogen, suggesting that this ex-

posure has an important role in the cause of breast cancer. Although a number of environmental chemicals are suspected of contributing to breast cancer, no single environmental chemical has been identified as a strong "smoking gun" for causing breast cancer (6). However, a consequence of excessive estrogen exposure may include unwanted cell division. The standard paradigm providing a general mechanistic explanation for the association of cumulative, excessive estrogen exposure and breast cancer risk was aptly stated by Feigelson and Henderson (4) and is shown in Fig. 1. The notion is that the proliferative stimulus provided by 17 β -estradiol (E_2) leads to the appearance of spontaneous mutations; thus, the key contribution of E_2 is the stimulation of breast epithelial cell proliferation (Chapter 8). However, an important aspect of estrogen toxicology is its tissue-specific, cellular oxidative metabolism by several specific cytochrome P450 isoforms and various peroxidases (7-10). Mounting evidence suggests that the oxidative metabolites may contribute to estrogen carcinogenesis (Chapters 4 and 5).

Among the metabolites formed during the process of estrogen biotransformation and elimination (Fig. 2), some are estrogenic (11) and some may be protective through their antioxidant properties and/or growth and angiogenesis inhibitory activities (12-14). On the other hand, the more reactive quinone metabolites are able to form direct adducts with DNA (15) and/or can cause oxidative damage to lipids (16) and DNA through redox cycling processes that produce reactive oxygen species (ROS) [(7, 8); Chapter 4]. Increased production of ROS could also lead to disruption of cellular redox homeostasis and, as a consequence, could alter transcription factor function, causing inappropriate alterations in the regulation of gene expression (17).

The possible contribution of these metabolites to estrogen carcinogenesis has received relatively little attention compared with that given to estrogen receptor-mediated processes. However, accumulating evidence, much of which was presented in this symposium, supports an expansion of the standard mechanistic paradigm for the causal association of estrogen exposure and breast cancer (Fig. 1). Thus, while estrogen-induced cell proliferation undoubtedly has an important role in estrogen carcinogenesis, complementary pathways involving indirect and/or direct genotoxicity originating from estrogen metabolites, in particular, the 4-hydroxy catechol metabolite, are also likely to make important contributions. Furthermore, since other metabolites, such as 2-methoxy E_2 , may have protective effects, a balance among these metabolites is likely required to maintain homeostasis.

In this chapter, I will provide a brief overview of some evidence in support of a role for estrogen metabolites in estrogen

Correspondence to: James D. Yager, Ph.D., Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, 615 North Wolfe St., Baltimore, MD 21205 (e-mail: jyager@jhsph.edu).

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Standard Paradigm

Estrogen, and perhaps progesterone "...affect the rate of cell division and thus manifest their effect on the risk of breast cancer by causing proliferation of breast epithelial cells. Proliferating cells are susceptible to genetic errors during DNA replication which, if uncorrected, can ultimately lead to a malignant phenotype."
(Feigelson and Henderson, *Carcinogenesis*, 17:2279-84, 1996)

Modified Paradigm

While estrogen-induced cell proliferation undoubtedly has important role in the carcinogenic process, mounting evidence supports a complimentary pathway involving:

Indirect and direct genotoxicity originating from estrogen metabolites, i.e. 4-OHE₂

•**Indirect:** Oxidative DNA damage via Redox Cycling → ROS

•**Direct:** Estrogen-quinone DNA adducts

•**Protective effects:** Perhaps through 2-methoxy catechol estrogen-mediated growth inhibition, apoptosis and anti-angiogenesis

Fig. 1. Standard and modified paradigms for estrogen carcinogenesis.

carcinogenesis. I will place particular emphasis on their potential for causing oxidative DNA damage in association with the carcinogenic process [see also two reviews (7,8)].

ESTROGEN OXIDATIVE METABOLISM

A more specific scheme for the oxidative metabolism of E₂ is shown in Fig. 3. The chemical structures of the estrogen catechols, semiquinones, quinones, and DNA adducts are presented in Chapter 4. The oxidative metabolites that have been shown to exhibit estrogenic and genotoxic effects include 16 α -hydroxyestrone [which will not be considered further in this monograph, but see (18–20)] and the 2-hydroxy- and 4-hydroxyE₂/estrone (E₁) catechols (7,8). The formation of the catechols is catalyzed by specific cytochrome P450 isoforms, including CYP1A1/1A2, CYP1B1, and CYP3A4 (7,8,21–24). The tissue specificity of estrogen metabolism results from the tissue-specific basal expression and inducibility of these enzymes and from differences among the P450 isoforms in their kinetic parameters for E₂ (23). This will be discussed in detail for CYP1A1 and CYP1B1 in Chapter 5 of this monograph, along with a discussion of the potential role for estrogen overproduction by aromatase in breast carcinogenesis.

ESTROGEN CARCINOGENESIS IN THE SYRIAN GOLDEN HAMSTER KIDNEY

Extensive evidence for a role for catechol estrogen (CE) metabolites in estrogen carcinogenesis has come from work done with the use of the male Syrian golden hamster kidney carcinogenesis model, principally in the laboratories of Liehr et al. (25) and Li and Li (26). The data in Table 1 demonstrate that E₂, E₁, and their 4-hydroxy CE (4-OHE₂ and 4-OHE₁), but not their 2-OH catechols, are carcinogenic in this model. Additional support for a role for the CEs was provided by the finding that quercetin, which is both a competitive and a noncompetitive inhibitor of the phase II enzyme catechol-O-methyltransferase (COMT), increased the number of large renal tumors and the incidence of abdominal metastases in E₂-treated hamsters (27,28). Much additional data support a role for CE metabolites in estrogen carcinogenesis in this model. For example, hamster kidney microsomes have been shown to biotransform estrogens to their 2-OHE₂ and 4-OHE₂ metabolites (29,30), whereas *in vivo* treatment caused the appearance of DNA strand breaks (31) and increased the levels of 8-hydroxy-deoxyguanosine (8-OHdG) (32). Han and Liehr (33) also reported that, using ham-

Fig. 2. Estrogens as carcinogens. ER = estrogen receptor; Ox = oxidative.

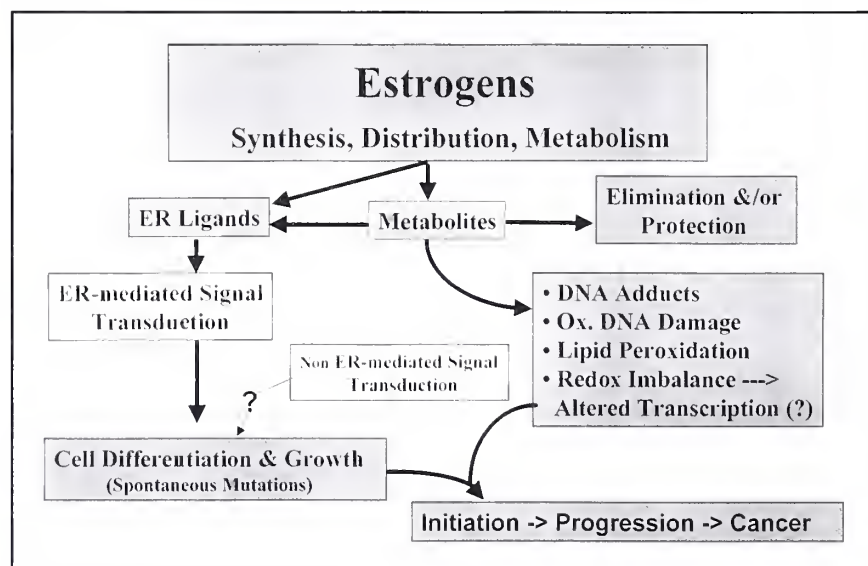


Fig. 3. Oxidative metabolism of estrogens. 2-OHCE and 4-OHCE = 2-hydroxy and 4-hydroxy catechol estrogens, respectively; COMT = catechol-*O*-methyltransferase; GSH = glutathione.

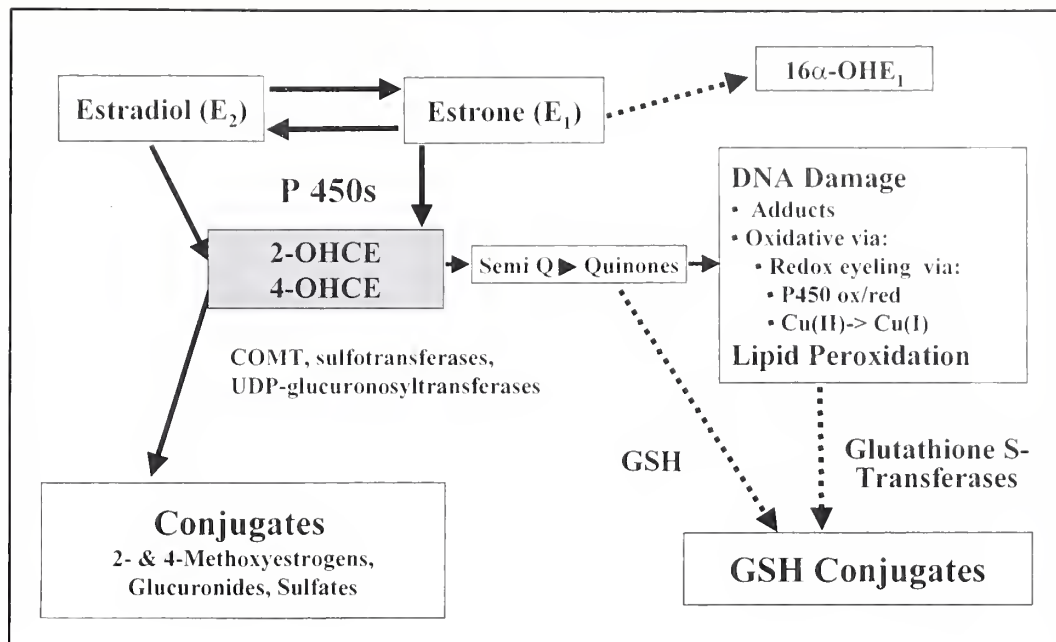


Table 1. Syrian golden hamster renal carcinogenicity of catechol estrogens

Estrogen*	% animals with renal carcinomas	
	Liehr et al. (25)	Li and Li (26)
None	0	0
E ₂	80	100
4-OHE ₂	80	100
2-OHE ₂	0	0
E ₁	—	80
4-OHE ₁	—	33
2-OHE ₁	—	0

*E₂ = 17β-estradiol; 4-OHE₂ = 4-hydroxyE₂; 2-OHE₂ = 2-hydroxyE₂; E₁ = estrone; 4-OHE₁ = 4-hydroxyE₁; 2-OHE₁ = 2-hydroxyE₁.

ster liver microsomes 4-OHE₂ but not 2-OHE₂ caused increased 8-OHdG levels. Similarly, Liehr and co-workers (34–36) demonstrated formation of 4-OHE₂ by microsomes from normal and tumor tissues of human breast, uterus, cervix, and ovary, whereas other investigators (37,38) have detected increased levels of 8-OHdG in human breast tumor tissue. These associations support the hypothesis that ROS generated through redox cycling processes involving CE metabolites may contribute to estrogen carcinogenesis of the human breast and perhaps other tissues.

ESTROGEN CARCINOGENESIS IN RAT LIVER

A role for oxidative DNA damage originating from estrogen metabolism is also supported by results from studies on the mechanisms of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and ethinyl estradiol (EE) carcinogenesis in rat liver. TCDD is a potent carcinogen in certain laboratory animals (39). Tritscher et al. (40) reported detecting higher levels of 8-OHdG in nuclear DNA from livers of TCDD-treated intact rats than in nuclear DNA from livers of TCDD-treated ovariectomized rats. Intact female rats are more sensitive to TCDD-induced hepatocarcinogenesis than are ovariectomized female rats or male rats (39,40). Since TCDD is a potent inducer of the P450s involved in the oxidative metabolism of E₂, these results are consistent with a

contribution of endogenous estrogens and increased oxidative DNA damage arising from estrogen metabolites in TCDD carcinogenesis in female rat liver, although other unknown mechanisms could be contributing to or could be responsible for this carcinogenic process in this experimental model.

Prolonged exposure of women to EE in the form of oral contraceptives has been associated with increased risk for developing hepatic tumors (41). A number of laboratories have been involved in studies on the mechanisms of EE hepatocarcinogenesis. The effects of EE on rat liver are summarized in Fig. 4 (7). EE is a strong promoter of hepatocarcinogenesis initiated by diethylnitrosamine, enhancing the development of altered hepatic foci, nodules, and carcinomas (7). In non-nitrosamine-initiated rats, EE alone is a weak carcinogen. Associated with this carcinogenic process, EE causes a transient increase in DNA synthesis, followed by growth inhibition, which provides a period of negative selective pressure during which resistant hepatocytes (initiated) begin clonal expansion (promotion) (42). In addition, increased oxidative DNA damage occurs during EE treatment, as shown by data (Table 2) compiled from those presented in a report by Ogawa et al. (43). In that study, female Wistar rats were treated with EE at the doses shown, which were sufficient to cause the development of hepatocellular carcinomas after 12 months. These results show an association between increased 8-OHdG and increased incidence of carcinomas. Furthermore, simultaneous treatment with each of three antioxidant vitamins inhibited tumor development and reduced the levels of oxidative DNA damage.

SUPPORT FROM MOLECULAR EPIDEMIOLOGIC STUDIES FOR A ROLE OF ENDOGENOUS CE METABOLITES IN HUMAN BREAST CANCER DEVELOPMENT

Evidence from model experimental systems mentioned above and described in the following chapters supports a role for CE metabolites in estrogen carcinogenesis. In humans, however, the growing body of available evidence is indirect and, thus, is only suggestive. As mentioned above, Liehr and Ricci (35) found 4-hydroxylase activity in normal and tumor breast tissues, Sutter

Fig. 4. Effects of ethinyl estradiol on rat liver. Adapted from Fig. 3 in (7). Reprinted with permission from the *Annual Review of Pharmacology and Toxicology*, Vol. 36, ©1996 by Annual Reviews www.AnnualReviews.org.

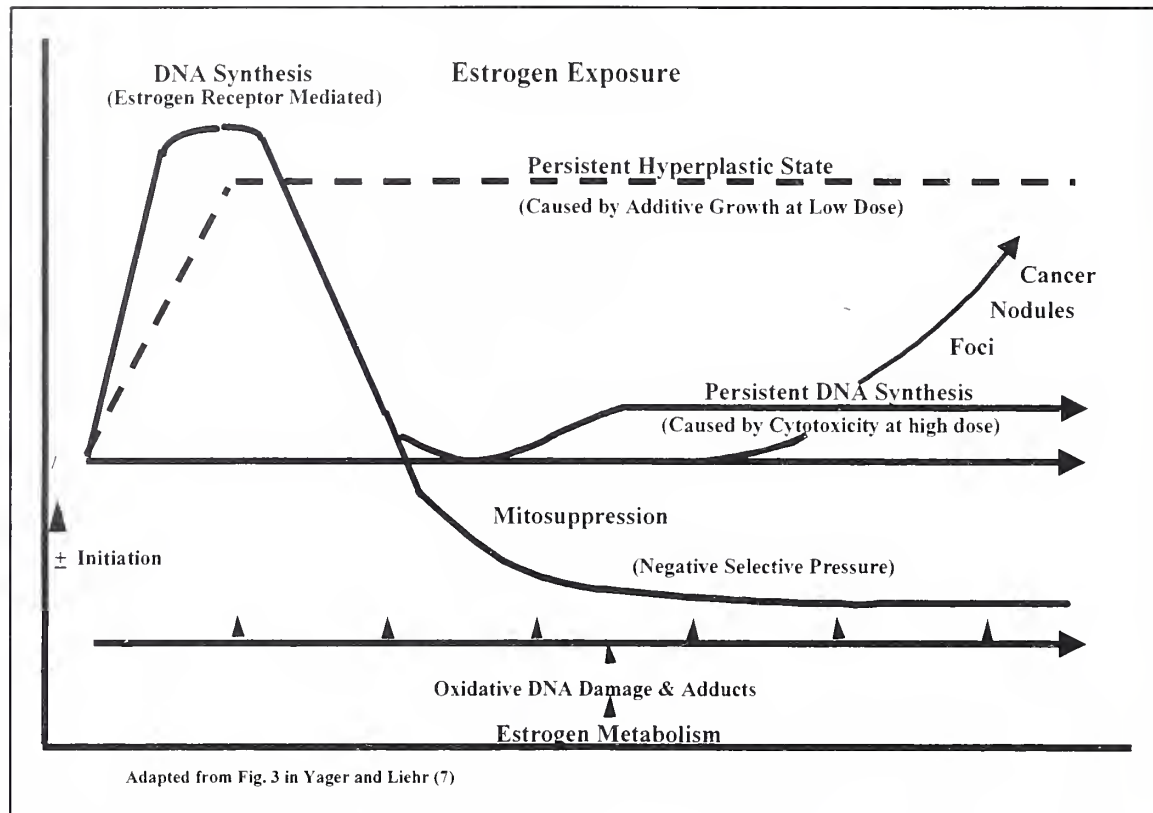


Table 2. Enhanced oxidative DNA damage in liver nuclear DNA from ethinyl estradiol (EE)-treated rats*

Treatment	8-oxodG/10 ⁶ dG, 1 mo, mean \pm standard deviation	Hepatocellular carcinoma incidence, 12 mo, % (No./total No.)
Control	3.5 \pm 0.7	0 (0/24)
EE, 75 μ g/day	7.1 \pm 0.9 [†]	8.7 (2/23) [†]
EE, 750 μ g/day	8.4 \pm 0.7 [†]	38.5 (10/26) [†]
EE, 75 μ g/day + vitamin C, 1g/kg diet	6.0 \pm 1.7	0 (0/19)
Vitamin E, 500 mg/kg diet	5.4 \pm 1.9	4.5 (1/22) [‡]
β -Carotene, 250 mg/kg diet	5.5 \pm 1.8	4.8 (1/21) [‡]

*Adapted from Ogawa et al. (43). oxodG = oxodeoxyguanosine; dG = deoxyguanosine.

[†] $P < .05$ versus control.

[‡] $P < .05$ versus EE alone.

(see Chapter 5) has observed expression of a 4-hydroxylase, CYP1B1, in human breast tissue, and Malins (37); Chapter 9) has detected increased oxidative DNA damage in breast tumor tissue. In addition, the results from some molecular epidemiology studies also provide support for a contribution of CE metabolites to the development of breast cancer. A number of studies, guided by the hypothesis that increased breast cancer risk is associated with exposures to certain environmental chemicals, have examined the association between risk and genetic polymorphisms in several genes encoding biotransformation enzymes. These include genes encoding CYP1A1 (44–46), *N*-acetyltransferase 2 (47), and glutathione-*S*-transferase (GST) isoforms M1 (null), T1 (null), and P1 (low-activity allele) (45,46,48). The results have been mixed, depending on the subject cohort. COMT is a gene involved in the phase II metabolism

of catechols, such as catecholamines and flavanoids. However, COMT also catalyzes the *O*-methylation of both 2-OH- and 4-OH-catechols formed from the oxidative metabolism of endogenous E₂ and E₁. *O*-Methylation of CEs inactivates their estrogenic potential and blocks their ability to undergo further oxidation to more reactive semiquinone and quinone metabolites that can directly adduct DNA and/or participate in redox cycling to produce superoxide, as described above. COMT is polymorphic, and 25% of Caucasians are homozygous for an allele encoding a low-activity form of the enzyme. The ability of CE metabolites to contribute to estrogen carcinogenesis, suggested by the experimental studies mentioned above and in Chapters 4 and 5 of this monograph, led to the hypothesis that women homozygous for the low-activity COMT allele would be at increased risk for breast cancer. At the time of this conference, two studies, one published (49) and another one that was in press but is now published (50), presented evidence that the gene encoding a low-activity form of COMT was associated with an increased risk for developing breast cancer in certain women. These data will be discussed in greater detail in Chapter 7.

Briefly, in a prospective, nested, case-control study from a large western Maryland cohort, Lavigne et al. (49) found that the risk for developing postmenopausal breast cancer associated with the low-activity COMT allele was increased in postmenopausal women who were also either heavy (body mass index >24.27 kg/m²) or GSTM1 null or GSTP1 low activity. In a hospital-based case-control study of subjects from western New York, Thompson et al. (50) found an increased breast cancer risk associated with the low-activity COMT allele, but only in premenopausal women, and they found that the risk was increased further in those women who had increased body mass indices (>23 kg/m²). Two additional studies have been published. In one study (51), no increase in risk was detected; in contrast, in the

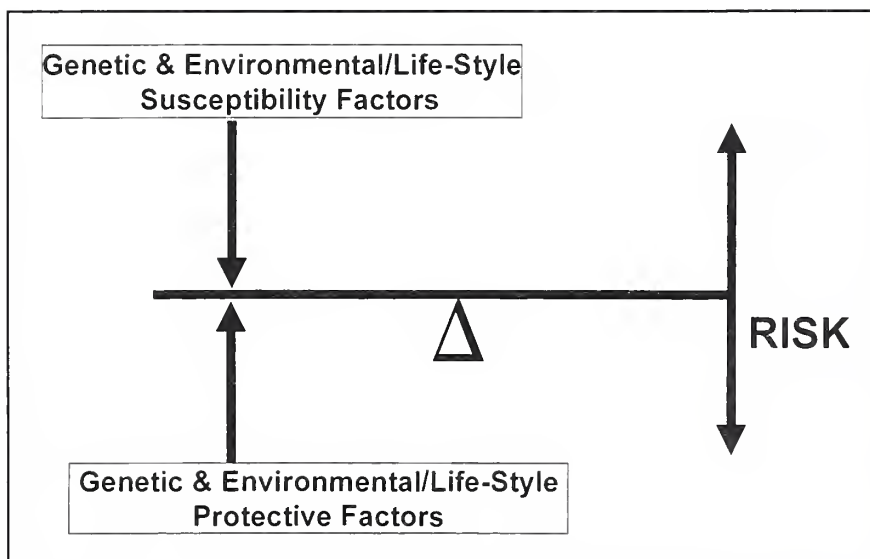
other study (52), an increased breast cancer risk associated with the low-activity COMT allele was found in postmenopausal women.

Why might there be such differences in the risk conferred by the same genetic polymorphism in different cohorts? One possibility is that these studies simply detected random findings. At this point, this possibility cannot be ruled out, for the number of cases and controls in these studies was small. On the other hand, the studies were hypothesis driven, and an increased risk associated with the low-activity allele has now been detected in three of four studies, one in premenopausal and two in postmenopausal women. Another possibility to account for the differences among the various cohorts may relate to the strength of the effect. According to a classification by Rebbeck et al. (53), mutations (detected as genetic polymorphisms) in some genes, such as BRCA1, confer a high risk for an individual. However, because these allele frequencies are low, the overall attributable risk that they represent is small. On the other hand, mutations in phase I and II enzyme genes involved in xenobiotic (but also endobiotic, i.e., endogenous molecules) metabolism might confer a low relative cancer risk for an individual. But, because these mutations seem to be common among individuals, they represent a high attributable risk category of genes. In addition, the specificities of many of these enzymes are overlapping, their activities within the cells can be altered by environmental agents, their polymorphic allele frequencies within a population can differ depending on ethnicity (54), and they function in somewhat redundant pathways. Thus, the balance in the cell of the metabolite products of these enzymes could be differentially affected by interactions among various genetic and environmental factors, as illustrated in Fig. 5. The intent of this scheme is to show that both genetic and environmental/lifestyle factors can act either as susceptibility or as protective influences with regard to risk of developing a particular disease. With regard to the oxidative metabolism and conjugation of estrogens, several enzymes are involved, including specific cytochrome P450 isoforms, sulfotransferases, COMT, and GST (for the products of oxidative damage). The levels of these enzymes in a given individual or population may be influenced (induced or inhibited) by xenobiotic exposures. In addition, these enzymes are polymorphic, and the distribution of polymorphisms may vary

among different populations as a result of ethnic compositions. Since the oxidative metabolites of estrogen appear to contribute to and protect from disease, it is, therefore, not surprising to detect differences in single-gene genetic polymorphism/disease associations among different study populations.

In summary, most of the data from the studies cited above and to be discussed in the following chapters that implicate metabolites of endogenous estrogens in estrogen carcinogenesis are from *in vitro* studies and *in vivo* studies in which rodent models were used. The results have provided important insight that has led to the development of a new paradigm (see Fig. 1) for the contribution of CE metabolites to estrogen carcinogenesis. It predicts that the level of and balance among the parent hormone and the catechol metabolites should be determined by the level of expression and activity of certain key enzymes including aromatase (Chapter 5), several cytochrome P450s, particularly CYP1B1 (Chapter 5), and various protective enzymes, such as COMT (Chapters 6 and 7). Expression of these enzymes is likely to be tissue specific as well as developmental stage specific and to be affected by both environmental and endogenous factors. These parameters need to be thoroughly defined. Expression levels should be determined, along with the levels of the estrogen metabolites and selected biologic end points for their potential effects, e.g., gene expression, DNA damage, and mutagenesis. For several of these enzymes, genetic polymorphisms that affect activity have been discovered; e.g., the COMT polymorphism decreases enzyme activity. The effects of these polymorphisms on estrogen metabolite levels and on the biologic end points also require thorough investigation. However, an important question pertains to the appropriate experimental model systems to use. One can envision using cultured human cells originating from various human tissues, e.g., breast, prostate, and ovary, along with cells genetically engineered to express these enzymes or their polymorphic forms alone and in combination. One can also envision using knockout mice to determine the effects of the absence of particular genes, e.g., aromatase, CYP1B1, and COMT, on tissue estrogen metabolite levels and on the biologic end points including cancer. From the knockout mice, it should be possible to create transgenics expressing the human genes. By use of bacterial artificial chromosome (bac) vectors that accept up to 300-kilobase inserts, it is

Fig. 5. Genetic, environmental, and lifestyle factors affect risk.



possible to include most of the regulatory regions of these genes and, thus, perhaps achieve their tissue-specific and developmental stage-specific expression. Results from studies such as these should more directly test the paradigm for the role of the CE metabolites. However, data obtained from human tissues will ultimately be required to be certain that the conclusions drawn from the model systems apply. Thus, efforts should be devoted to obtaining normal and tumor human tissues, e.g., breast and prostate, for simultaneous analysis of estrogen metabolites and the relevant biologic end points, e.g., DNA damage, CYP1B1 genotype and expression, and COMT genotype and expression. It will only be through such future in-depth mechanistic studies that we will be able to dissect the genetic and environmental "factors" affecting the pathways that provide adverse or protective "states," characterized by a correct or incorrect balance of oxidative metabolites impacting cancer risk. Identification of these factors should also allow more focused, mechanistically based molecular epidemiologic studies to be conducted in the future.

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NOTES

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Chapter 4: Estrogens as Endogenous Genotoxic Agents—DNA Adducts and Mutations

Ercole Cavalieri, Krystyna Frenkel, Joachim G. Liehr, Eleanor Rogan, Deodutta Roy

Estrogens induce tumors in laboratory animals and have been associated with breast and uterine cancers in humans. In relation to the role of estrogens in the induction of cancer, we examine formation of DNA adducts by reactive electrophilic estrogen metabolites, formation of reactive oxygen species by estrogens and the resulting indirect DNA damage by these oxidants, and, finally, genomic and gene mutations induced by estrogens. Quinone intermediates derived by oxidation of the catechol estrogens 4-hydroxyestradiol or 4-hydroxyestrone may react with purine bases of DNA to form depurinating adducts that generate highly mutagenic apurinic sites. In contrast, quinones of 2-hydroxylated estrogens produce less harmful, stable DNA adducts. The catechol estrogen metabolites may also generate potentially mutagenic oxygen radicals by metabolic redox cycling or other mechanisms. Several types of indirect DNA damage are caused by estrogen-induced oxidants, such as oxidized DNA bases, DNA strand breakage, and adduct formation by reactive aldehydes derived from lipid hydroperoxides. Estradiol and the synthetic estrogen diethylstilbestrol also induce numerical and structural chromosomal aberrations and several types of gene mutations in cells in culture and *in vivo*. In conclusion, estrogens, including the natural hormones estradiol and estrone, must be considered genotoxic carcinogens on the basis of the evidence outlined in this chapter. [J Natl Cancer Inst Monogr 2000;27:75–93]

Estrogens, including the natural hormones estradiol (E_2) and estrone (E_1), induce tumors in various organs of several laboratory animal species and strains [reviewed in (1,2)]. In humans, exogenous estrogen-containing medications or elevated concentrations of circulating endogenous estrogens increase the risk of uterine and mammary cancers [reviewed in (1,2)]. Nevertheless, synthetic or steroidal estrogens or their metabolites failed to induce gene mutations in several classical bacterial and mammalian gene mutation assays (3–7) and were, therefore, classified as epigenetic carcinogens (8,9). Two possible mechanisms of tumor induction by estrogens were subsequently advanced. Estrogen-induced cell transformation and tumor development were proposed to be mediated by 1) estrogen receptor-based proliferation of cells carrying spontaneous replication errors [(8,10); Chapter 8] and 2) disruption of spindle formation and subsequent numeric chromosomal changes (9).

An increasing body of experimental evidence stands, however, in contradiction to these two hypotheses of hormonal tumorigenesis, including the following data: 1) In human mammary epithelial cells, estrogen receptors are expressed in cells different and distinct from proliferating cells carrying proliferation markers (11,12). 2) Aneuploidy and other karyotypic changes were detected in Syrian hamster embryo cells predisposed to immortalization and progression to tumorigenicity;

nude mice inoculated with cells carrying such chromosomal alterations, however, did not produce tumors (13). Therefore, additional genetic changes (mutations) were postulated to be required for tumor induction (13). 3) Compared with hamsters treated only with E_2 , tumor formation is decreased in animals exposed to E_2 plus inhibitors of estrogen metabolism (14,15) or to hormonally potent estrogens with poor metabolic conversion to catechol metabolites (16,17). These data support a tumor-initiating role for catechol estrogens (CE). 4) A large body of evidence is accumulating that estrogens induce various types of DNA damage *in vitro* and *in vivo*. As outlined below, CE can, indeed, mediate this damage. 5) The classification of estrogens as epigenetic carcinogens is contradicted by preliminary evidence of estrogen-induced gene mutations (reviewed below).

In this chapter, we discuss the induction of DNA damage and gene mutations. First, we focus on the direct adduction of estrogen metabolites to DNA *in vitro* and *in vivo*, second on the generation of reactive oxygen species (ROS) by estrogen metabolites and various types of DNA damage induced indirectly by estrogens and, finally, on estrogen-induced gene mutations.

ONCOGENIC MUTATIONS BY DEPURINATING CARCINOGEN—DNA ADDUCTS AS A MODEL OF ESTROGEN-INDUCED MUTATIONS

The origin of cancer represents one of the most intriguing scientific mysteries. Cancer is a disease of mutated critical regulatory genes and abnormal cell proliferation (18). Understanding the origin of these mutations opens the door to strategies for controlling and preventing cancer. One possible approach to investigating the origin of cancer has been to gain a fundamental understanding of the properties of molecules that induce this disease. During the last 25 years, polycyclic aromatic hydrocarbons (PAH) have been investigated by Cavalieri and Rogan (19,20) as model carcinogenic compounds. The purpose of studying these molecules has been threefold: First, they represent a good model for understanding the mechanism of tumor initiation by chemicals; second, they have some geometric resemblance to endogenous estrogens; and third, both PAH and estrogens contain aromatic rings.

Affiliations of authors: E. Cavalieri, Eppler Institute, University of Nebraska Medical Center, Omaha, NE; K. Frenkel, New York University School of Medicine, NY; J. G. Liehr, Stehlin Foundation for Cancer Research, Houston, TX; E. G. Rogan, Eppler Institute, University of Nebraska Medical Center; D. Roy, University of Alabama at Birmingham, School of Public Health.

Correspondence to: Ercole Cavalieri, D.Sc., Eppler Institute for Research in Cancer and Allied Diseases, 986805 Nebraska Medical Center, Omaha, NE 68198-6805 (e-mail: ecavalie@unmc.edu).

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Comprehensive studies of PAH have led to an understanding of their mechanism of tumor initiation (19,20). PAH are activated by two main pathways: one-electron oxidation to produce reactive intermediate radical cations and monooxygenation to afford bay-region diol epoxides (19,20). The reactive intermediates formed by these two mechanisms, radical cations and diol epoxides, can bind to DNA to produce adducts that initiate the process of tumor formation, as illustrated in Fig. 1 for dibenzo[*a,l*]pyrene (DB[*a,l*]P). DNA adducts are obtained by reaction of the metabolically activated PAH with the nucleophilic groups of the two purine bases, adenine (Ade) and guanine (Gua). These adducts can be either stable or depurinating. The stable adducts are those that remain covalently bonded to DNA unless removed during repair, whereas the depurinating adducts are the ones that are spontaneously released from DNA by destabilization of the glycosidic bond (Fig. 2). Stable DNA adducts are formed when PAH react with the exocyclic amino group of Ade or Gua, whereas depurinating adducts are obtained when PAH covalently bond at the N-3 or N-7 position of Ade or the N-7 or, sometimes, the C-8 position of Gua.

Among the various approaches to the study of carcinogenesis by PAH, identification and quantitation of their DNA adducts

have been the most fruitful in unraveling the mechanism of tumor initiation by these compounds. Through comprehensive studies of the DNA adducts of the potent carcinogenic PAH, benzo[*a*]pyrene (BP), 7,12-dimethylbenz[*a*]anthracene (DMBA), and DB[*a,l*]P (Fig. 3), Cavalieri and Rogan (20) and Chakravarti et al. (21) have discovered that there is an association between depurinating adducts and oncogenic mutations, suggesting that these adducts are the primary culprits in the tumor initiation process. This discovery was made by identifying and quantifying the DNA adducts formed in mouse skin by BP, DMBA, and DB[*a,l*]P and, at the same time, determining the mutations in the Harvey (H)-ras oncogene in mouse skin papillomas initiated by these three PAH, as shown in Fig. 4 (21). When mouse skin was treated with DMBA, 79% of the adducts were depurinating Ade adducts and 20% were depurinating Gua adducts (20,22). For DB[*a,l*]P, 81% were depurinating Ade adducts and 18% were depurinating Gua adducts (20). In contrast, mouse skin treated with BP produced 46% depurinating Gua adducts and 25% depurinating Ade adducts (20,23). Examination of the ras oncogene mutations in papillomas induced by DMBA or DB[*a,l*]P demonstrates that, in both cases, an A → T transversion (CAA → CTA) consistently occurs (Table 1; Fig. 5) (21). These mutations associate with the predominant formation of depurinating Ade adducts by these two PAH. About twice as many of the papillomas induced with BP contain G → T mutations at codon 13 in ras (GGC → GTC) compared with the number of tumors with a codon 61 CAA → CTA mutation (21,24). The ratio of mutations is consistent with the profile of depurinating Gua and Ade adducts formed by BP in the target tissue (Table 1). This pattern of ras mutations suggests that the oncogenic mutations in mouse skin papillomas induced by these PAH are generated by misreplication or misrepair of the apurinic sites derived from loss of the depurinating adducts (21). For example, an A → T transversion can be attributed to loss of a depurinating Ade adduct and generation of an apurinic site. If the apurinic site is not correctly repaired in the next round of DNA replication, the most likely base to be inserted opposite the apurinic site is Ade (Fig. 6). When the coding strand of the DNA is then replicated, a thymine is inserted opposite the new Ade, resulting in the A → T mutation observed in codon 61 of the ras oncogene in tumors initiated by PAH forming predominantly depurinating Ade adducts. When a Gua adduct is lost by depurination, leaving an apurinic site in the DNA, the preferential insertion of Ade in the opposite DNA strand leads to a G → T transversion at the site of the adduct.

It is also possible that the ras mutations are generated by misrepair, rather than misreplication, of the apurinic sites. Strong evidence for misrepair is provided by the observation of codon 61 CAA → CTA transversions in mouse skin DNA 1 day after treatment with DB[*a,l*]P (Table 2) (25), when the cells are unlikely to have divided. The A → T transversions are present in 0.1% of the cells by day 1, increase to about 5% by day 3, and then decrease to background levels by day 9. Subsequently, the A → T mutation is detected in increasing levels as papillomas begin to develop.

Because thousands of apurinic sites are spontaneously formed per cell each day, repair of apurinic sites induced by PAH might be expected. The level of apurinic sites arising from treatment with PAH is, however, 15–120 times higher than those formed spontaneously, suggesting that this large increase in apurinic sites could overwhelm the capacity of the cell to repair them

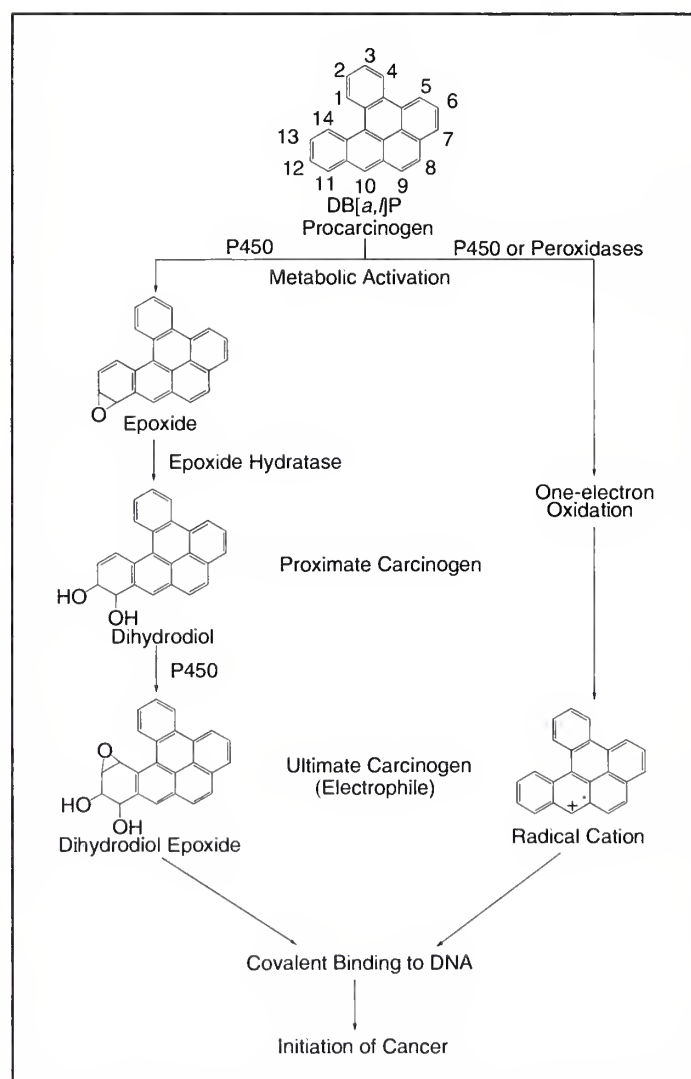
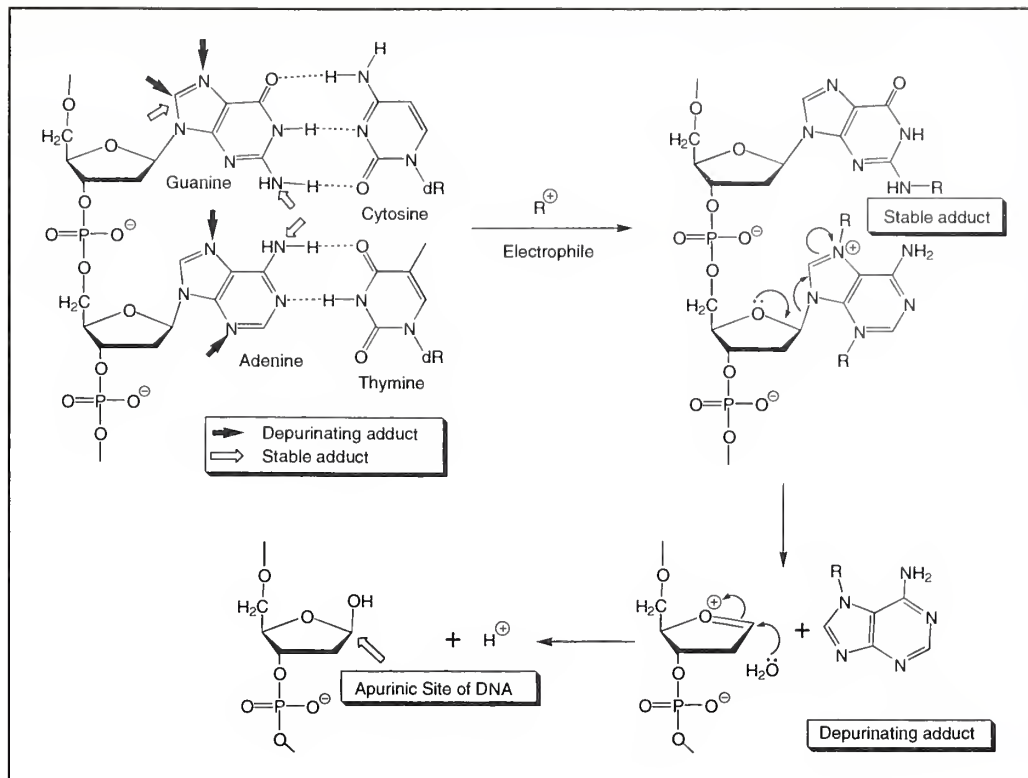


Fig. 1. Metabolic activation of DB[*a,l*]P by the diol epoxide and radical cation pathways.

Fig. 2. Formation of stable and depurinating DNA adducts and generation of apurinic sites.



before replication occurs (20,21). Furthermore, the apparent nonrepair of apurinic sites induced by treatment with PAH may also be due to the presence of stable adducts that could interfere with error-free repair of apurinic sites. Thus, apurinic sites can generate the mutations that play the critical role in the initiation of cancer, and formation of depurinating adducts has become the common denominator for recognizing the potential of a chemical to initiate cancer.

The evidence that depurinating PAH-DNA adducts play a major role in tumor initiation has provided the impetus for discovering the estrogen metabolites that form depurinating DNA adducts and can be potential endogenous initiators of many human cancers (26,27).

CATECHOL ESTROGEN-3,4-QUINONES AND APURINIC SITES IN CANCER INITIATION

CEs are among the major metabolites of E_1 and E_2 , as discussed in Chapter 5. If these metabolites are oxidized to catechol estrogen quinones (CE-Q), they may react with DNA to form depurinating adducts. It is hypothesized that these adducts generate apurinic sites leading to mutations, which may initiate breast, prostate, and possibly other human cancers. The estrogens E_1 and E_2 are biochemically interconvertible by the enzyme 17 β -estradiol dehydrogenase. These two estrogens are metabolized via two major pathways: formation of CE (Fig. 7) and, to a lesser extent, 16 α -hydroxylation (not shown). The catechols formed are the 2-hydroxylated and 4-hydroxylated estrogens (28,29). Generally, these two CEs can be inactivated by *O*-methylation catalyzed by catechol-*O*-methyltransferases (COMT) (28). Other possible conjugations of CE, such as glucuronidation and sulfation (not shown), may also play a role in inactivation/protection (Chapter 6). If formation of the 4-hydroxylated metabolites is excessive (*see below*) and/or production of these methyl, glucuronide, or sulfate conjugates is insufficient and, thus, the cells are not totally protected from CE toxicity, competitive catalytic oxidation to semiquinones (CE-SQ) and CE-Q can occur. CE-SQ and CE-Q may conjugate with glutathione (GSH), catalyzed by *S*-transferases. If this inactivating process is incomplete, CE-Q may react with DNA to form stable and depurinating adducts (26,27).

To determine the possible genotoxic effects of CE-Q, they were reacted with the nucleosides 2'-deoxyguanosine (dG) and 2'-deoxyadenosine (dA) and the nucleobase Ade (26,30). An acetonitrile solution of E_1 (E_2)-3,4-Q was mixed with dG, dissolved in acetic acid/water (1:1) (Fig. 8). The adduct 4-OHE₁(E_2)-1(α , β)-N7Gua was formed during 5 hours at room

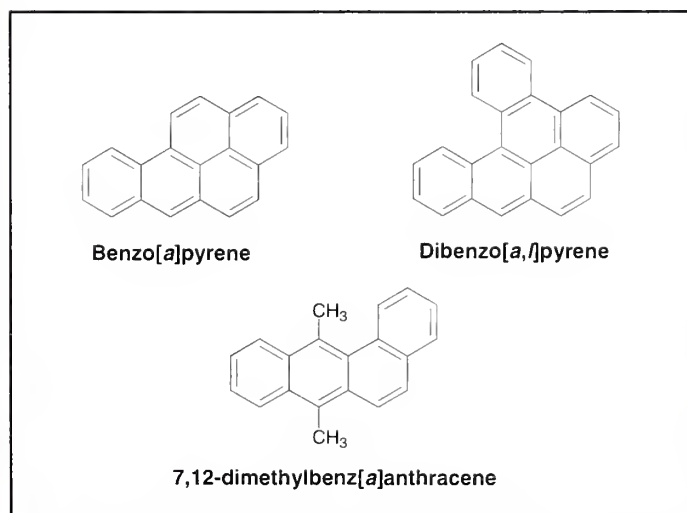


Fig. 3. Structures of three potent carcinogenic polycyclic aromatic hydrocarbon.

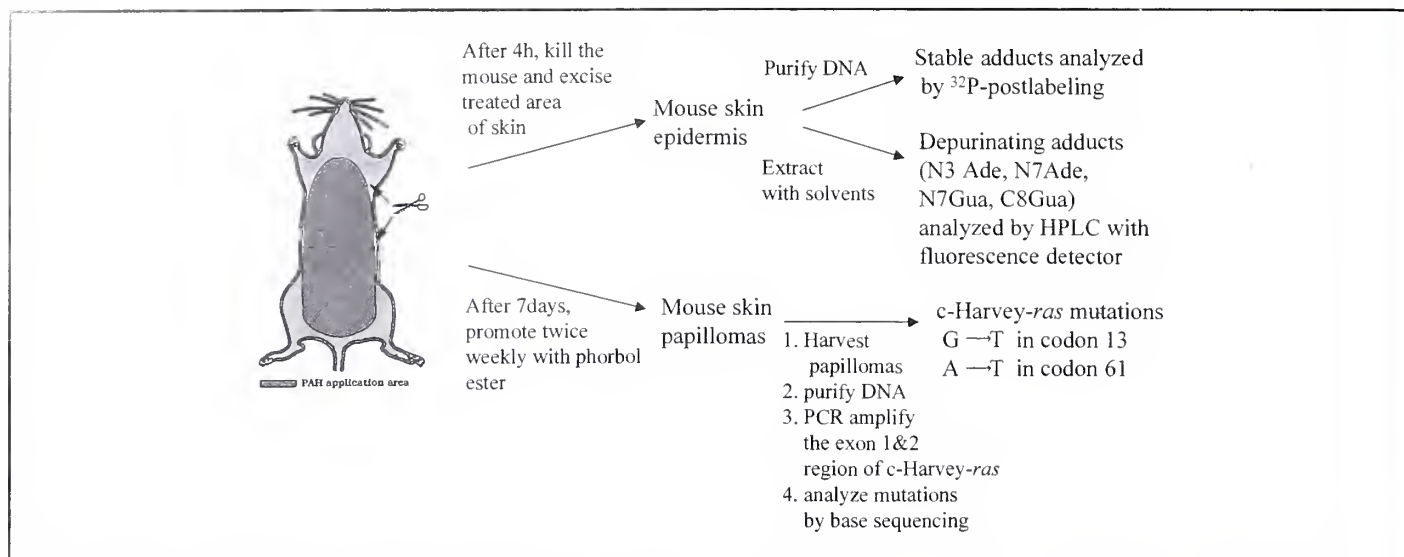


Fig. 4. Determination of DNA adducts and Harvey-ras mutations in mouse skin.

Table 1. Correlation of depurinating adducts with H-ras mutations in mouse skin papillomas*

PAH	Major DNA adducts (%)	H-ras mutations	
		No. of mutations/ No. of mice	Codon
DMBA	N7Ade (79)	4/4 CAA → CTA	61
DB[a,l]P	N7Ade (32) N3Ade (49)	4/5 CAA → CTA	61
BP	C8Gua + N7Gua (46)	10/20 GGC → GTC	13
	N7Ade (25)	5/20 CAA → CTA	61

*PAH = polycyclic aromatic hydrocarbons; DMBA = 7,12-dimethylbenz[a]anthracene; DB[a,l]P = dibenzo[a,l]pyrene; and BP = benzo[a]pyrene.

temperature (26), and it is a mixture of two conformational isomers resulting from the restricted rotation of the Gua moiety around the N7(Gua)-C1(estrogen) bond. The reaction of the CE-3,4-Q with dG at the N-7 position destabilizes the glycosidic bond and results in loss of the deoxyribose moiety. When the adduct is formed by reaction of CE-3,4-Q with DNA, it is released from the DNA by spontaneous depurination. Reaction of $E_1(E_2)$ -3,4-Q with dA produced no adducts; however, reaction of $E_1(E_2)$ -3,4-Q with Ade resulted in the formation of 4-OHE₁(E₂)-1(α,β)-N3Ade (Fig. 8) (30). This adduct was obtained only with Ade because in dA the adjacent deoxyribose bonded to N-9 impedes the approach of the electrophile $E_1(E_2)$ -3,4-Q to N-3 (23,31). This interference is not present in DNA, as evidenced by formation of PAH-N3Ade adducts, which are rapidly lost from the DNA by depurination (23,32).

When E_1 -2,3-Q reacted with dG or dA, a profile of adducts totally different from those formed by E_1 -3,4-Q was obtained (Fig. 9) (26). Reaction of E_1 -2,3-Q with dG afforded 2-OHE₁-6-N⁶dG and with dA yielded 2-OHE₁-6-N⁶dA. In this case, the E_1 -2,3-Q did not react as a quinone, but as its tautomer, the E_1 -2,3-Q methide. This electrophile reacts at C-6 with the exocyclic amino group of dA or dG to yield the N⁶dA and N⁶dG adducts, which retain the deoxyribose and are referred to as "stable" adducts because they remain in DNA unless repaired.

To determine whether these adducts are formed in biologic systems, E_2 -3,4-Q or enzymically activated 4-OHE₂ was reacted with DNA for 2 hours at 37°C (Fig. 10). The stable adducts were determined by the ³²P-postlabeling method, and depurinating adducts were analyzed by high-performance liquid chromatography (HPLC) interfaced with an electrochemical detector (27). When E_2 -3,4-Q reacted with DNA, almost the same amount of 4-OHE₂-1(α,β)-N7Gua and 4-OHE₂-1(α,β)-N3Ade were obtained (Table 3). The amount of stable adducts was 0.02% of the depurinating adducts. Activation of 4-OHE₂ with horseradish peroxidase gave similar results, whereas lactoperoxidase produced a similar amount of N3Ade adduct but about 50% more N7Gua adduct (Table 3). The same two depurinating adducts were obtained in equal but smaller amounts when 4-OHE₂ was activated with phenobarbital-induced rat liver microsomes (cytochrome P450) (27).

When female Sprague-Dawley rats were treated by intramammary injection of 4-OHE₂ or E_2 -3,4-Q, the 4-OHE₂-1(α,β)-N7Gua adduct was detected in the mammary tissue (27). The N3Ade adduct presumably was also present, but its synthetic standard was not available at the time of the study. These data clearly show that CEs are enzymatically oxidized to CE-Q and bind to DNA *in vitro* and *in vivo*.

Several additional lines of evidence suggest that oxidation of 4-hydroxyestrogens is the pathway leading to estrogen-induced cancer. 4-Hydroxyestrogen formation has been observed to predominate in hamster kidney (33,34) and other organs prone to estrogen-induced tumors, such as rat pituitary (35) and mouse uterus (36). In fact, 4-hydroxyestrogens induce kidney tumors in male Syrian golden hamsters, whereas the 2-hydroxyestrogens do not (4,37). Predominant 4-hydroxylase activity has also been found in human microsomes of uterine myometrium and benign uterine leiomyomas (38) as well as in microsomes of benign and malignant breast tumors (39,40). In tissues resistant to estrogen-induced tumors, such as the liver, formation of 2-hydroxyestrogens predominates (39). Furthermore, 2,3,7,8-tetrachlorodibenzo-p-dioxin induces cytochrome P450 1B1, which predominantly catalyzes hydroxylation of E_2 at the C-4 position (41-44). The importance of this finding is related to evidence that exposure to dioxin greatly increases the risk of developing cancer (42,45).

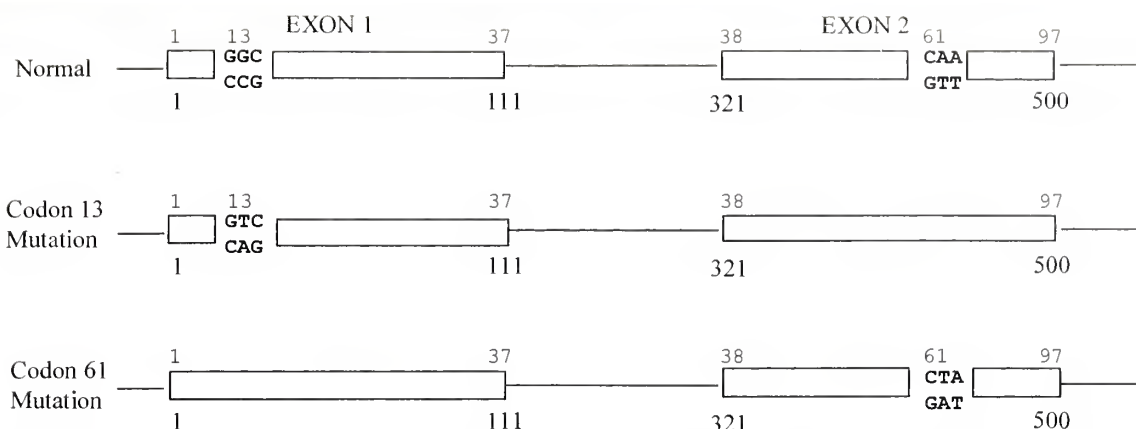


Fig. 5. Mouse Harvey-ras mutations. The normal Harvey-ras proto-oncogene (**top**) can be activated by mutation at codon 61 (CAA → CTA, **middle**) or codon 13 (GGC → GTC, **bottom**).

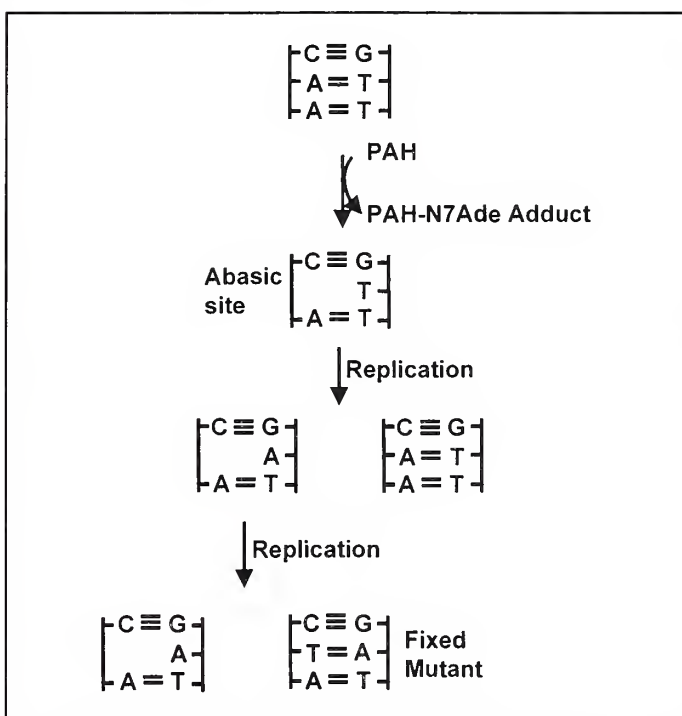


Fig. 6. Possible scheme for inducing A → T mutations from depurinating Ade adducts.

The combination of increased formation of the 4-hydroxylated CEs and their oxidation to CE-3,4-Q, which react with DNA to form the depurinating adducts associated with oncogenic mutations, suggests that the 4-hydroxyestrogen pathway producing CE-3,4-Q is responsible for the genotoxic effects leading to estrogen-induced initiation of cancer.

The kidney of male Syrian golden hamsters is an established model for estrogen-induced tumorigenesis (46,47). Two hours after hamsters were given an injection intraperitoneally with 4-OHE₂, the kidneys were removed and extracts were analyzed for the adducts formed by 4-OHE₂ with DNA and GSH by using HPLC with electrochemical detection and confirmation by mass spectrometry (48). With DNA, the predominant adducts are 4-OHE₂-1(α,β)-N7Gua and 4-OHE₂-1(α,β)-N3Ade; only the

Table 2. Frequency of the Harvey-ras mutation in codon 61 (CAA → CTA) after treatment of mouse skin with dibenzo[a,l]pyrene

Time after treatment, days	% of H-ras genes with codon 61 mutations
0	<0.001
1	0.1
2	1
3	5
6	0.5
9	<0.001
35	0.1
63 (tumors)	14–47

*Data taken from (25).

N7Gua adduct was analyzed because different gradient conditions would have been needed for the N3Ade adduct. With GSH, 4-OHE₂ forms the 4-OHE₂-2-SG conjugate (49,50). This conjugate is further metabolized to 4-OHE₂-2-cysteine and 4-OHE₂-2-N-acetylcysteine by the mercapturic acid biosynthesis pathway. Therefore, all of these conjugates were searched for, along with the 4-OHE₂-1(α,β)-N7Gua adduct. Preliminary results indicate that 4-OHE₂-1(α,β)-N7Gua and all of the GSH-derived conjugates are present in the kidney 2 hours after injection of 4-OHE₂, with the cysteine conjugates being the most abundant. As hypothesized for the initiation of cancer by estrogens, these results demonstrate that, in the hamster kidney, 4-OHE₂ is oxidized to E₂-3,4-Q, which binds to GSH and to DNA, forming depurinating adducts.

The nonsteroidal estrogen hexestrol, which is diethylstilbestrol (DES) hydrogenated at the C-C double bond, is carcinogenic in Syrian golden hamsters (46,51). The major metabolite of hexestrol and DES is their catechol (51–54), which can be metabolically converted to their catechol quinone. This hexestrol quinone has chemical and biochemical properties similar to those of CE-3,4-Q, i.e., it specifically forms an N7Gua adduct after reaction with dG or DNA (55). These data suggest that the hexestrol catechol quinone is the electrophile involved in tumor initiation by hexestrol. In turn, these results substantiate the hypothesis that CE-3,4-Q may be endogenous tumor initiators.

In conclusion, the pathway of activation, i.e., oxidation of estrogens to CE and then to CE-Q, affords the ultimate carci-

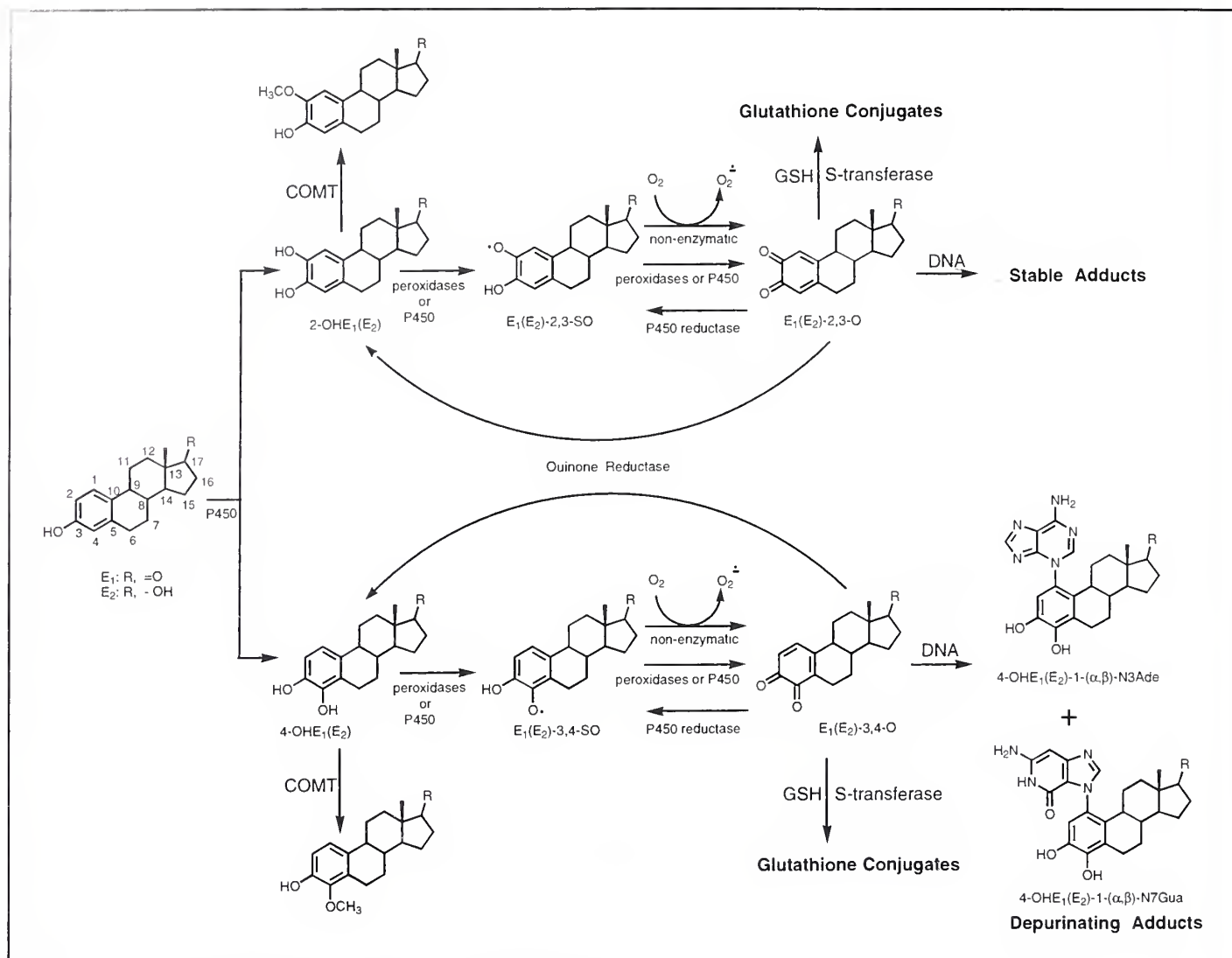


Fig. 7. Activating and deactivating (protecting) pathways of estrogen metabolism and formation of DNA adducts.

nogenic metabolites that are CE-3,4-Q for endogenous estrogens and catechol quinones for synthetic estrogens. This competitive, oxidative pathway takes place only when excessive formation of 4-CE and/or their incomplete inactivation occur. The DNA damage by these reactive electrophiles consists of the formation of depurinating adducts and apurinic sites in DNA. Misrepair and/or misreplication of the apurinic sites in DNA may generate the critical mutations that trigger induction of cancer by estrogens (21,25).

ESTROGEN-MEDIATED FORMATION OF OXIDANTS AND OXIDATIVE DNA DAMAGE: THEIR ROLE IN CARCINOGENESIS

Oxidants are continuously formed and degraded in normal cellular processes. They are necessary for a plethora of biochemical reactions, without which life itself could not be sustained. Cells are equipped with extensive multilayer antioxidant defenses to intercept excess oxidants. However, when ROS are generated at an inappropriate time, in excessive amounts, or when antioxidant defenses are overwhelmed, then the negative effects of oxidants become apparent. In the following presentation, we will concentrate on the damaging effects of oxidants

induced endogenously by estrogens and their putative role in the carcinogenic process.

One of the major types of damage that oxidants directly induce is oxidative modification of the genetic material. There are well over 30 different types of oxidized bases that can be formed in DNA; their levels exceed those of the stable carcinogen-induced adducts with DNA bases by about two orders of magnitude, being on average $1/10^5$ versus $1/10^7$ normal DNA bases, respectively (56,57). Some of these oxidized DNA bases are mutagenic (58–60) and induce DNA hypomethylation (61,62), a process known to increase gene expression (63).

The oxidized base derivatives most frequently discussed in this presentation are 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 5-hydroxymethyl-2'-deoxyuridine (HMDu) (for structures see Fig. 11). As oxidized bases are formed in DNA, various types of repair enzymes start removing them (64,65). There is always a background level of oxidized bases present in DNA, which seems to be tolerated by the cells. However, conditions leading to a continuous elevation of oxidized bases in DNA are the same as those that induce tumor promotion processes.

The following examples illustrate that target sites for estrogen carcinogenesis invariably also contain elevated levels of oxidized bases in cellular DNA. Conversely, the presence of higher

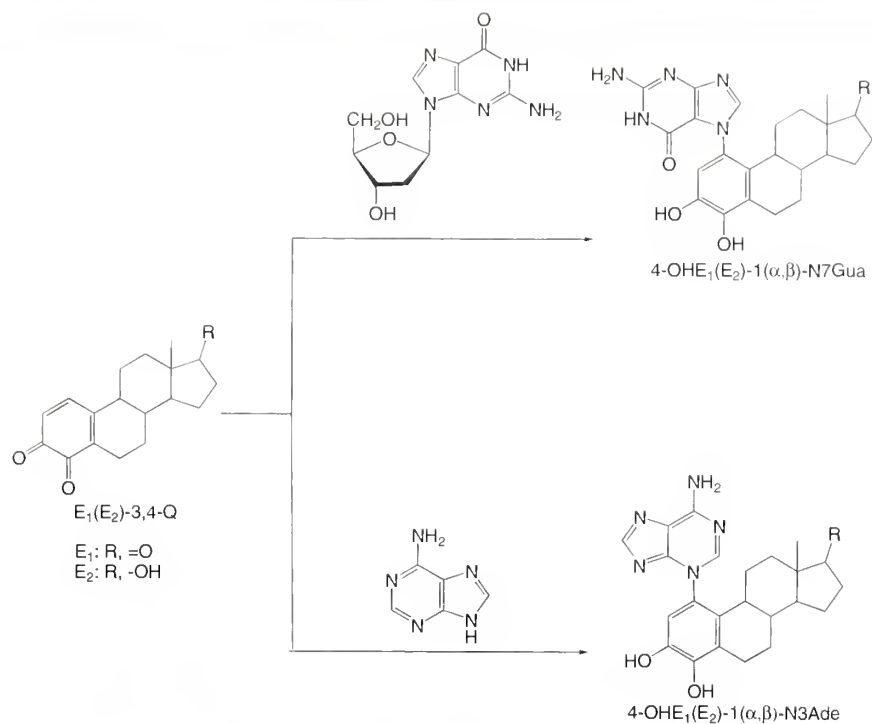


Fig. 8. Reaction of $E_1(E_2)\text{-}3,4\text{-Q}$ with dG or Ade.

than normal amounts of oxidized DNA bases may be indicative of a carcinogenic process induced by estrogens.

Oxidized DNA Bases as Evidence of Endogenous Oxidant Formation by Estrogens

Animal models. The formation of either 8-OHdG or HMdU in the target tissues for estrogen-mediated carcinogenesis has

repeatedly been shown. The animal models often utilized are Syrian hamster kidney (66) and dorsolateral prostate in the Noble rat [(67); M. Bosland: unpublished data]. For example, the highest 8-OHdG increase (sevenfold) occurred in the periurethral section of the dorsolateral prostate isolated from the Noble rat treated with testosterone and E_2 (Table 4; M. Bosland: unpublished data). This is the same part of the organ where

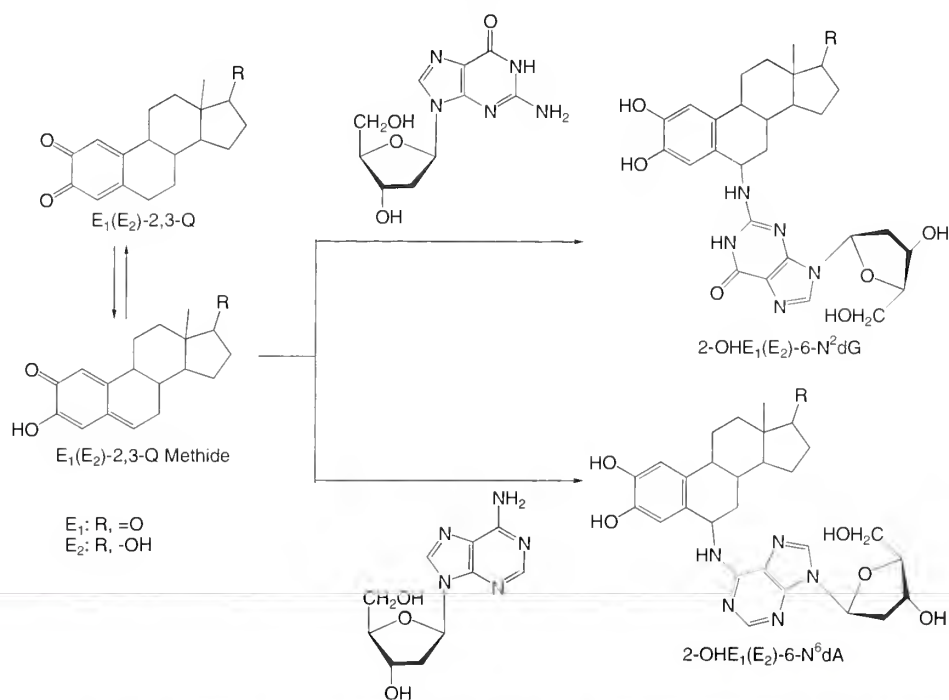


Fig. 9. Reaction of $E_1(E_2)\text{-}2,3\text{-Q}$ with dG or dA.

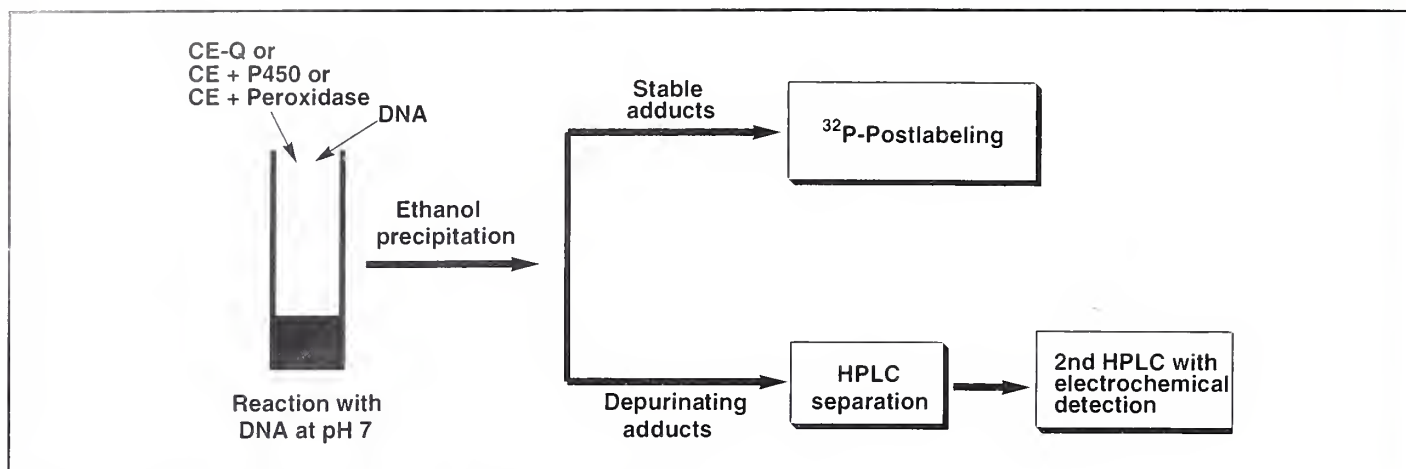


Fig. 10. Methodology of *in vitro* binding of CE-Q and activated CE to DNA.

Table 3. Reaction of E₂-3,4-Q and HRP-, LP-, or P450-activated 4-OHE₂ with DNA*

Compound	Depurinating adducts, $\mu\text{mol/mol DNA-P}$		Stable adducts, $\mu\text{mol/mol DNA-P}$	Ratio of depurinating to stable adducts
	4-OHE ₂ -1(α,β)-N7Gua	4-OHE ₂ -1(α,β)-N3Ade		
E ₂ -3,4-Q	186	196	0.06	6367
4-OHE ₂				
+ HRP	197	198	0.03	13 167
+ LP	363	208	0.05	11 420
+ P450	125	133	ND	

*HRP = horseradish peroxidase; LP = lactoperoxidase; P450 = cytochrome P450 in phenobarbital-induced rat liver microsomes; ND = not yet determined.

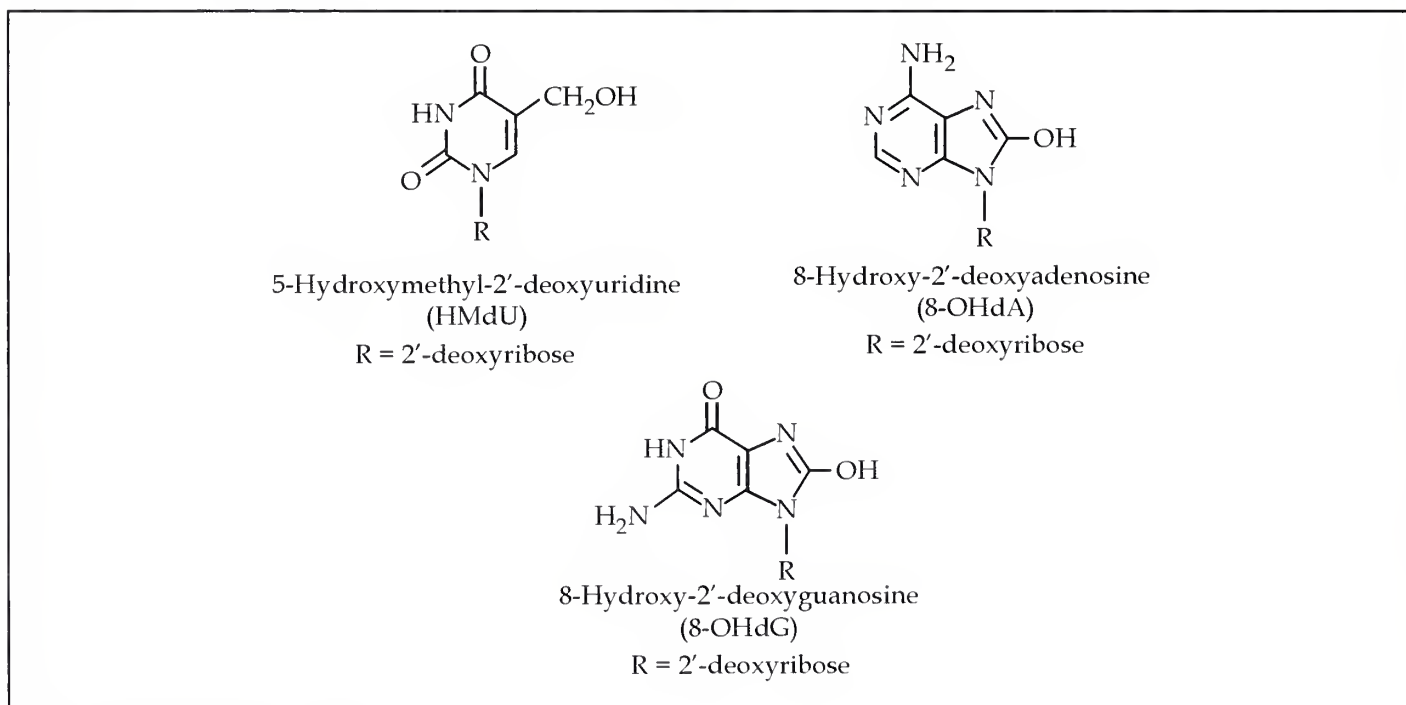


Fig. 11. Examples of oxidized DNA bases.

adenocarcinoma growth (83% of animals), DNA adduct formation (~fourfold increase), and lipid peroxidation (>threefold) occur (Chapter 2). There was no cancer growth, DNA adducts, or 8-OHdG formation in the periphery. Thus, the oxidative DNA damage was detected at the same selected tissue site (10% of the whole prostate) where adenocarcinoma develops.

Formation of 8-OHdG and HMdU was also significantly elevated and persisted in mouse skin topically treated with DMBA, a potent skin and mammary carcinogen, through the stages of tumor promotion and progression, and was evident at the time of tumor growth (Fig. 12) (68). The importance of oxidants and oxidative DNA damage in DMBA carcinogenesis

Table 4. Testosterone and estradiol-induced changes in dorsolateral prostate*

Endpoint measured	Periurethral			Periphery		
	Control	Treated	% Change	Control	Treated	% Change
Adenocarcinoma	0	10/12	83	0	0	
DNA adducts	2.7	10.2*	380	ND	ND	
Lipid hydroperoxides	1.4	4.5	320	2.0	3.8	190
8-OHdG	0.3	2.1†	700	ND	0.1	

*From (67) and M. Bosland; unpublished data.

†*P* = .02; ND = not detectable.

is underscored by the long-known fact that pretreatment with antioxidants causes a suppression of DMBA-induced tumors without affecting levels of stable DNA adducts (69). DMBA treatment evoked sustained, long-lasting inflammatory responses characterized by neutrophilic infiltration and edema (68). DMBA also enhances expression of IL-1 α messenger RNA (mRNA) and elevates the activity of IL-1 α (70). Estrogens, even at a low physiologic dose, also increase formation of this inflammatory cytokine (71), which, in turn, has a pronounced effect on a cascade of further inflammatory and carcinogenic responses (72–74).

Cellular models. Both HMdU and 8-OHdG are present in MCF-10A human breast epithelial cells (immortal but not tumorigenic) and in MCF-7 breast cancer cells (75,76). The basal levels of both modified bases are ~80% higher in MCF-7 cells, a finding that is expected because tumor cells produce substantial levels of hydrogen peroxide (H₂O₂), one of the major cellular oxidants (57,77,78). HMdU levels increased in response to H₂O₂ (75), and those of 8-OHdG, in response to the DMBA treatment (76). These increases are higher in MCF-10A cells and reach levels prevalent in tumor cells. Carcinogen treatment of MCF-10F cells (also immortal but otherwise a normal cell line) is known to lead to their malignant transformation (79). Hence, the increase in oxidized DNA bases likely occurs during the process of cellular transformation.

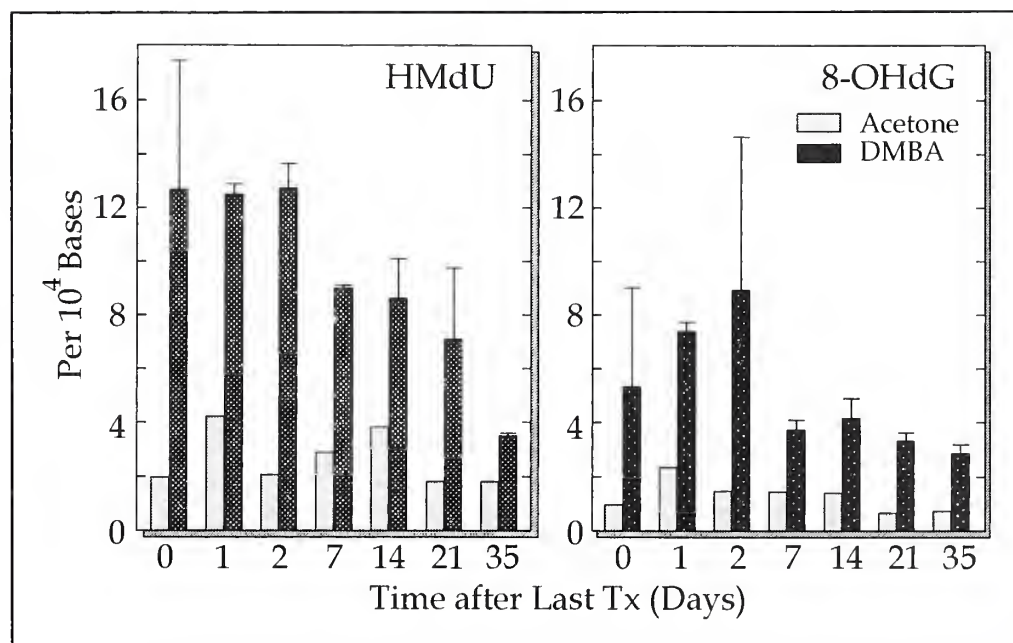
Humans. More important, HMdU was shown to be present in

white blood cell DNA of women at a high risk for breast cancer and those diagnosed with breast cancer (80,81). Of interest, a decrease in fat intake and presumed increase in vegetables and fruit consumption significantly decreased HMdU levels in women at high risk for breast cancer. In general, levels of oxidized purines were significantly elevated in human breast cancer. However, they were also increased even at sites distal to the cancerous tissue, not only in the breast, but also at other sites of hormonal carcinogenesis, such as ovarian and, in men, prostatic cancers [(82,83); Chapter 9]. In fact, it has been proposed that the increased levels of oxidized bases in human DNA precede cancer development and may serve as biomarkers of cancer risk (81,83,84). There is extensive evidence that chemical carcinogens generally induce formation of oxidized bases in DNA of target tissues *in vivo*. Estrogens are no exception and induce tumors by comparable mechanisms (57,66,85).

Mechanisms of Estrogen-Mediated Oxidant Formation

What are the sources of endogenous oxidants formed in response to estrogens? Estrogens, like other chemical carcinogens, are metabolized by cytochrome P450 enzymes and form hydroxylated products. The main metabolites of E₂ include 2-, 4-, and 16 α -hydroxyestradiol (Fig. 13) (33–36,44,86–88). The 2- and 4-hydroxylated catechols contain the hydroxyl groups in a vicinal position, which predisposes them to further oxidation. Both can be oxidized to semiquinones, which in the presence of molecular oxygen are oxidized to quinones with formation of superoxide anion radicals (O₂^{•-}), as illustrated in Fig. 7 (66,89,90). These O₂^{•-} readily dismutate to H₂O₂ either spontaneously or even faster when catalyzed by superoxide dismutase. H₂O₂ is neutral and rather nonreactive, except in the presence of the reduced transition metal ions (i.e., Fe²⁺ and Cu⁺), which cause formation of the most powerful and indiscriminate oxidants, hydroxyl radicals (•OH) (91,92). However, as a neutral molecule, H₂O₂ can readily cross the cellular and nuclear membranes and reach DNA in neighboring cells, where it can cause site-specific oxidation of bases (57). Quinones and semiquinones are capable of redox cycling as long as there is molecular

Fig. 12. Formation of HmdU and 8-OHdG in 7,12-dimethylbenz[*a*]anthracene (DMBA)-treated SENCAR mouse skin DNA. Adapted from (68).



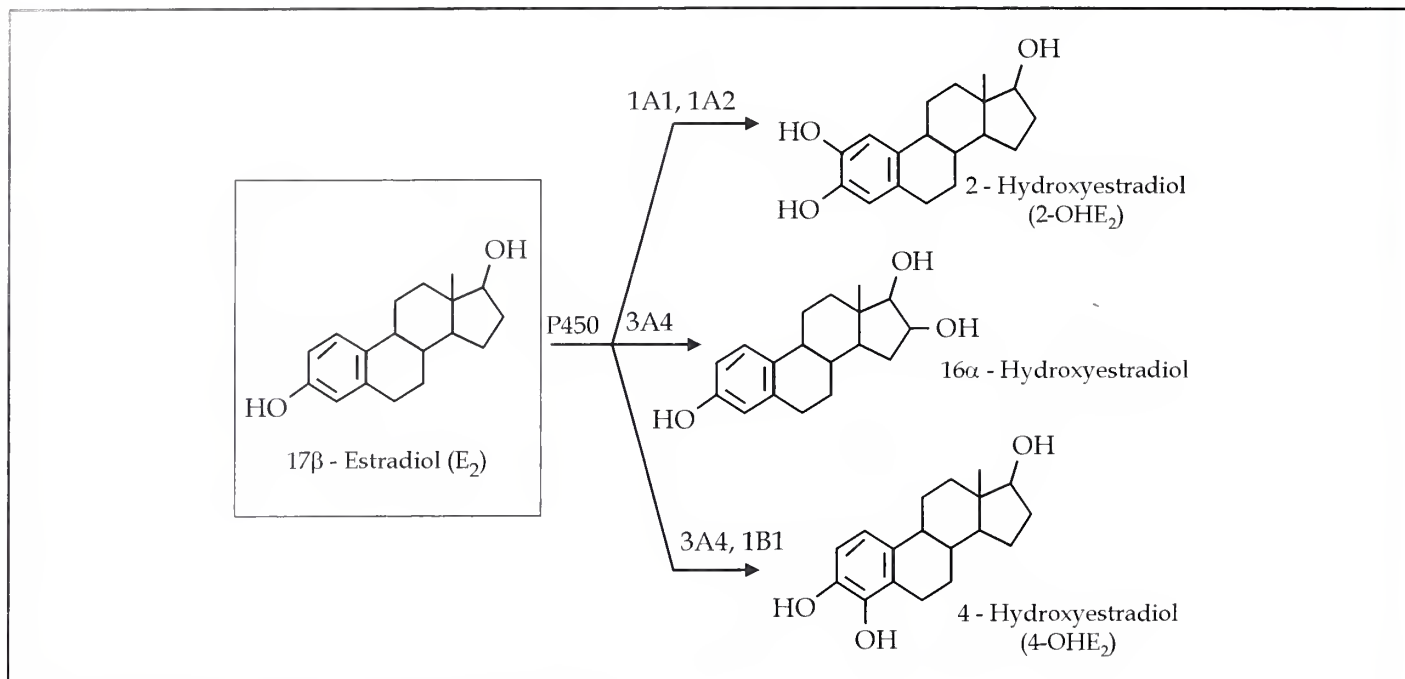


Fig. 13. Oxidative metabolism of E_2 . Adapted from (44) and (87).

oxygen available and, therefore, even a small amount of E_2 may cause substantial ROS production and subsequent cellular damage.

This ROS formation by redox cycling of semiquinones and quinones is mitigated by cellular quinone reductase, an enzyme that reduces quinones back to catechols by use of using reduced nicotinamide adenine dinucleotide (NADH) as a reducing co-factor. Moreover, COMT may prevent oxidation of CE to CE-SQ by methylating 2- or 4-hydroxyl groups (Fig. 14). However, it appears that the 4-hydroxyl group is not as readily methylated as is the 2-hydroxyl substituent, which results in the predominance of 4-OHE₂ in redox cycling, while 2-OHE₂ is virtually inactivated by methylation (93). Furthermore, methylated 2-OHE₂ was shown to inhibit COMT-mediated methylation of

4-OHE₂, which may allow for the accumulation of this carcinogenic metabolite in those organs where both metabolites are formed (93). The rapid methylation of 2-OHE₂ may be one reason for its lack of carcinogenic activity (4,37), whereas the lesser methylation of 4-OHE₂ contributes to its carcinogenic properties (4,37,39).

Like PAH, estrogens can generate ROS by peroxidatic metabolism (94). For example, E_2 was shown to produce phenoxyl radicals in the lactoperoxidase-catalyzed reaction. These phenoxyl radicals rapidly react with the cellular-reducing agents GSH and NADH. However, instead of detoxification, other radical species are formed (GS^\bullet and NAD^\bullet , respectively), which reduce molecular oxygen to $O_2^{\bullet-}$, followed by H_2O_2 formation

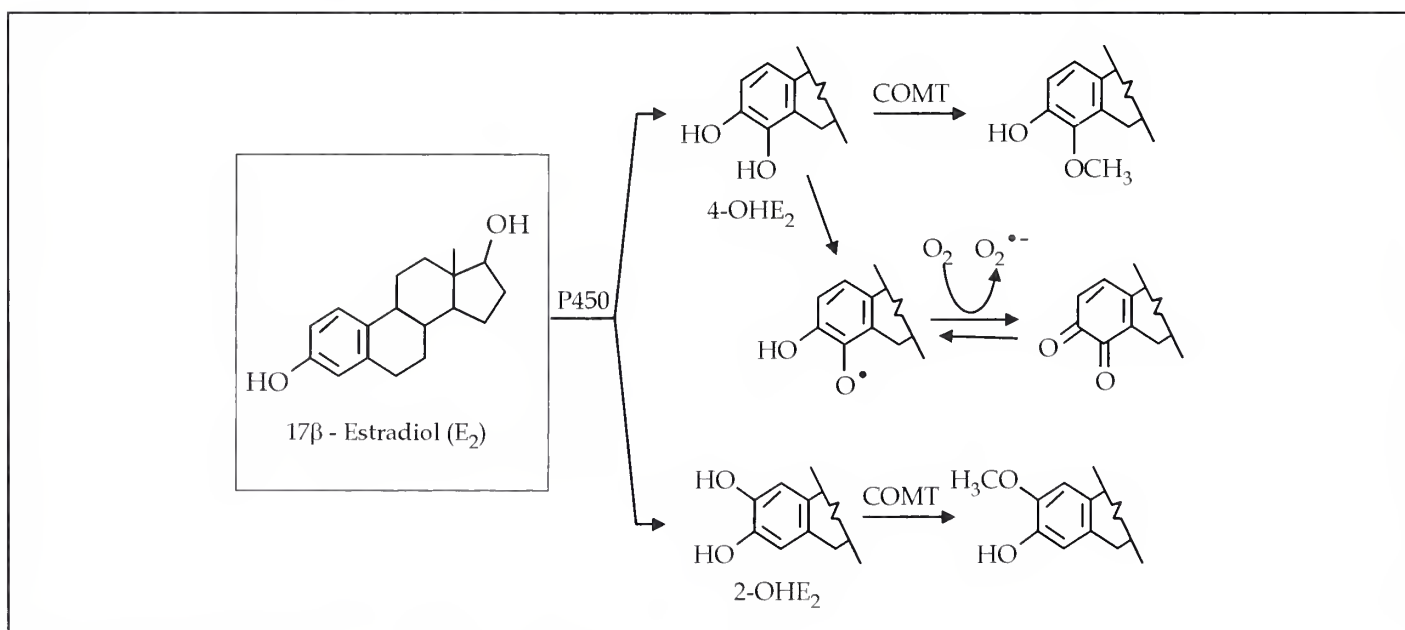


Fig. 14. Redox cycling of catechols.

(Fig. 15). The regenerated GSH and NADH can continue this process as long as O_2 is available. Of the cellular reductants tested, only ascorbate radical does not further react with O_2 , thereby breaking the radical chain that leads to ROS formation. Lactoperoxidase and estrogens are ubiquitously present in milk ducts and in the mammary gland (94).

Estradiol-Induced Lipid Peroxidation and DNA Damage

ROS may also cause oxidation of cellular macromolecules other than DNA, which include proteins and lipids. For example, oxidation of cysteine residues at an active site of an enzyme would either inactivate or at least change the activity of that enzyme (95). Many biosynthetic and energy-producing antioxidants as well as repair enzymes have redox-sensitive centers, which are readily modified by prooxidant changes (96). Hence, E_2 -induced ROS may have a pronounced effect on cell maintenance and functioning.

Lipids, particularly polyunsaturated lipids, are readily peroxidized, and the products often participate in a chain reaction propagating formation of various radical species (97). Lipid hydroperoxides are formed during prooxidant conditions generated by different sources, which include inflammation and carcinogen exposures. Again, like other carcinogens, estrogens induce lipid peroxidation during their metabolic activation (66). The insidiousness of this process is demonstrated by the fact that lipid hydroperoxides formed during E_2 metabolism may serve as cofactors in further E_2 (or other carcinogen) metabolism to hydroxylated products and in the oxidation of CE to quinone intermediates, which continuously amplifies the formation of lipid hydroperoxides and cellular damage (Fig. 16). Furthermore, lipid hydroperoxide-derived aldehydes, such as malondialdehyde and 4-hydroxynonenal, interact with bases in cellular DNA, thus increasing the burden of DNA modification (98,99).

An interplay between estrogen metabolites and oxidants leads to at least three types of DNA base damage (Fig. 16): DNA base adducts produced by quinones, as described above, lipid hydroperoxide-derived aldehyde DNA adducts, as well as a

plethora of oxidized DNA bases. The importance of ROS formation during estrogen metabolism is underscored by the fact that H_2O_2 generated by the redox cycling of the semiquinone-quinone couple is readily reduced by cellular transition metal ions, such as Fe^{2+} and Cu^+ , to hydroxyl radicals ($\bullet OH$), the most potent oxidants. Hydroxyl radicals not only oxidize bases in DNA, but also cause lipid peroxidation. Lipid hydroperoxides then serve as cofactors in further estrogen metabolism, which leads to additional semiquinone-quinone redox cycling, ROS production, and so on.

Estradiol-Mediated Modulation of Immune Responses

Although estrogens themselves can induce ROS production, they can also modulate immune responses and immune-mediated diseases, as indicated in Table 5, which can predispose to cancer (71,100). This process may take place under prooxidant conditions occurring in the course of inflammatory processes. In fact, at physiologic doses, E_2 potentially induces interleukin (IL)-1 α , a cytokine that can initiate a cascade of other cytokines, chemotactic and growth factors (71,101). Chemotactic factors cause infiltration of phagocytes, which may be activated to secrete a plethora of other cytokines, ROS, and reactive nitrogen species (RNS) (72,74,102–105). On the other hand, E_2 inhibits IL-1 α -induced IL-6 production. Therefore, by suppressing IL-6 formation, E_2 increases human epithelial cell proliferation, a process important in tumor growth, while it also inhibits the activity of natural killer cells, thus allowing tumor growth (101,105). E_2 mediates macrophage proliferation and decreases cell differentiation (71). Each of the affected processes contributes to the environment, allowing or encouraging tumor cell development and growth.

Estrogen Effects on Macrophages and Their Production of ROS and RNS

Macrophages have been found in normal human breast tissue. However, their numbers increase tremendously in breast tumors, providing up to 50% of the tumor mass (106,107). This increase in mass might be compounded by estrogen-stimulating macro-

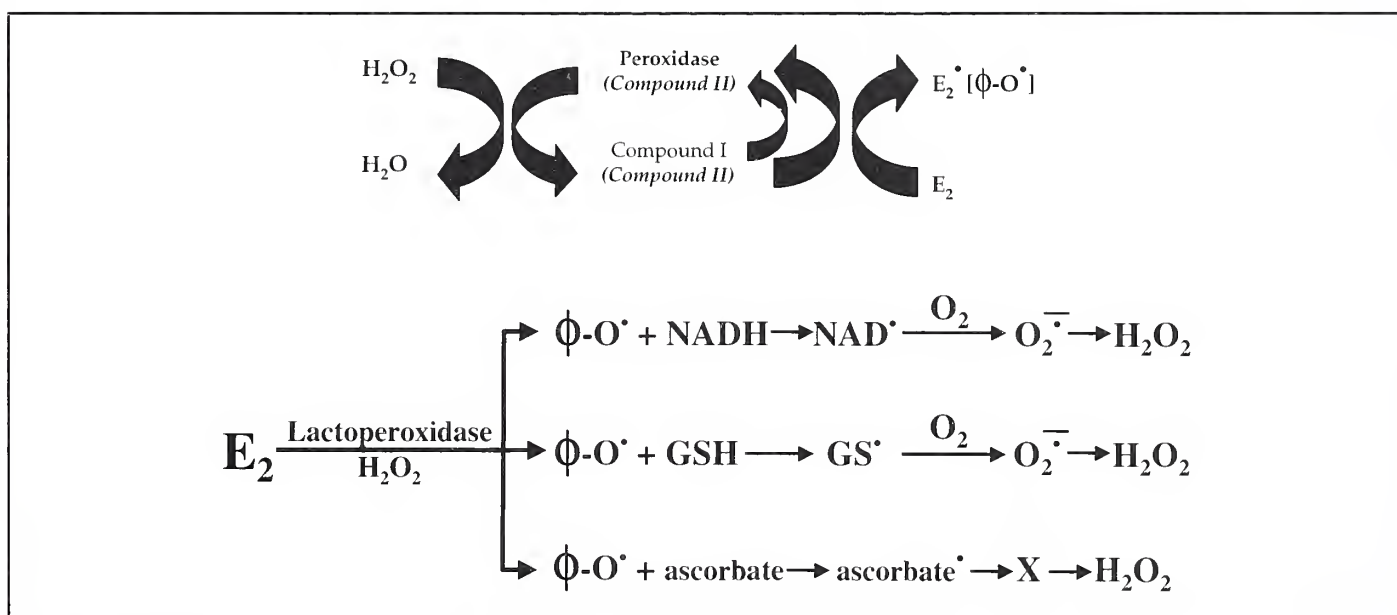


Fig. 15. Futile estrogen metabolism. Adapted from (94).

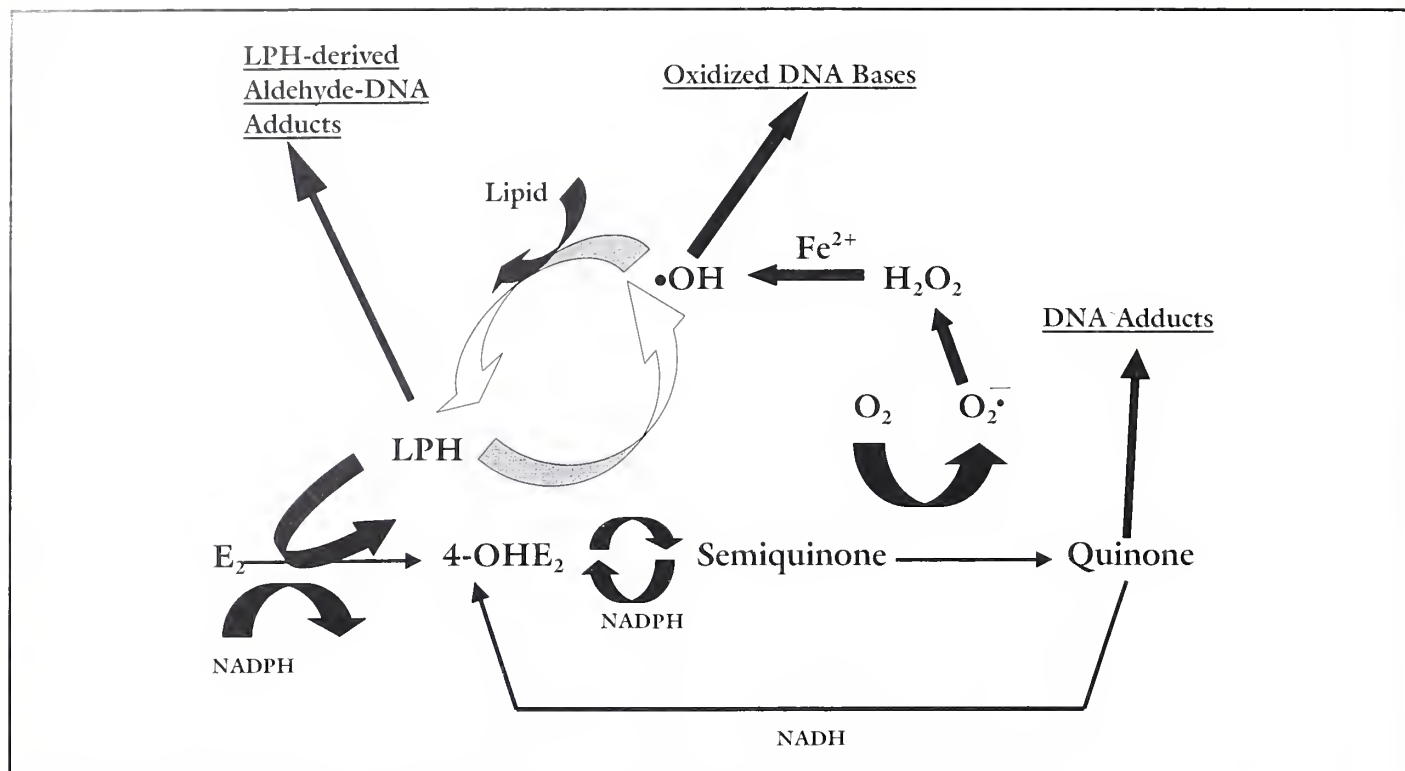


Table 5. Effects of estradiol (E₂) on immune responses*

*IL = interleukin; Ig = immunoglobulin.

On stimulation, macrophages produce oxidants such as $O_2^{\bullet -}$ and H_2O_2 by an reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase catalyzed reduction of molecular oxygen. This process occurs rapidly by the activation of the constitutive NADPH oxidase. A few hours after macrophage stimulation, inducible nitric oxide synthase is synthesized by the cells and mediates production of L-arginine-derived nitric oxide ($\bullet NO$), another radical species, which participates in signal transduction, numerous reactions, and cellular processes. $O_2^{\bullet -}$ and $\bullet NO$ may rapidly interact, with the evolution of peroxynitrite, a much more potent oxidant (110). Hence, macrophage activation may lead to ROS and RNS, which include $O_2^{\bullet -}$, H_2O_2 , $\bullet OH$, and singlet oxygen [1O_2 , known to oxidize dG to 8-OHdG (111)], peroxynitrite ($ONOO^{\bullet -}$), nitrite, nitrate, as well as nitrating species. Among them, ROS and RNS may cause

Estrogen Effects on PMN Function and ROS Formation

The estrogen-mediated action causes HOCl/OCl⁻ formation and ensuing oxidative cell damage, even in the absence of the proper targets. Moreover, estrogens can stimulate (by 10-fold) the activity of the released myeloperoxidase, thus compounding the damaging effects. The interaction of HOCl/OCl⁻ with an excess of H₂O₂ causes regeneration of chloride ions as well as evolution of species that can chlorinate and oxidize DNA (Fig. 17) (112,114).

Various interactions occur at physiological pH among reactive oxygen and nitrogen species generated by the phagocytic cells, macrophages and PMNs (Fig. 17). Those interactions lead to various types of DNA modification, many of which result in mutations. Therefore, estrogens, by modulating phagocytic cell

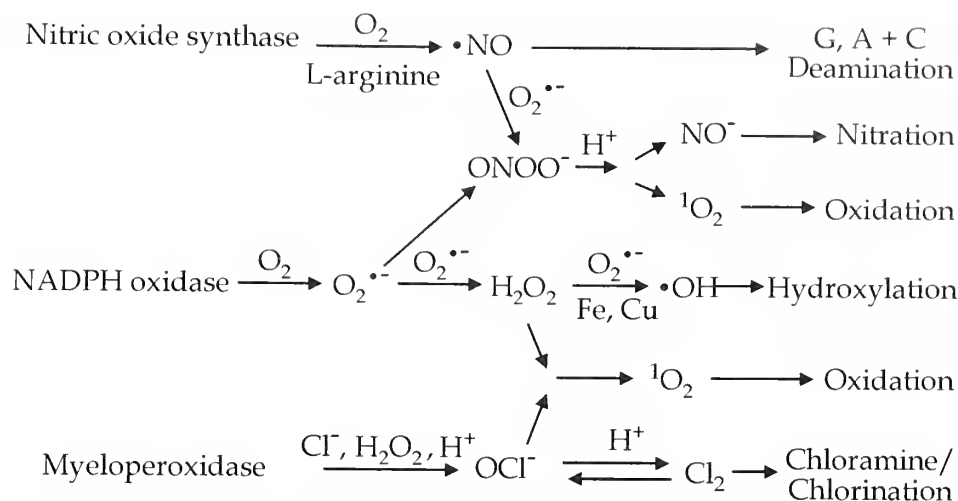


Fig. 17. Oxygen-derived, enzyme-driven major cellular ROS at physiologic pH. Summary of literature by Khan AU, Frenkel K.

proliferation and activation, have a pronounced effect on the integrity of DNA and mutagenesis.

ROS generated by PMNs and macrophages cause not only DNA modification but also oxidation of proteins and lipid peroxidation. As shown in Fig. 16, lipid hydroperoxides can now serve as cofactors of estrogen metabolism, during which ROS are produced, as well as other DNA-damaging species. Therefore, estrogens affect inflammatory responses and, in turn, their activities are affected by the inflammation products.

Immunomodulation by Estrogens

One of the more pronounced properties of E_2 is its ability to differentiate T and B cells, increase immunoglobulin production, and aggravate immune complex-mediated diseases, such as systemic lupus erythematosus, which occur predominantly in females (115–117). This disease is characterized by strong inflammatory responses, which are thought to contribute to it, as well as to various types of cancer (57,73,112,117).

Women at high risk of breast cancer, those with benign breast diseases, and those who are diagnosed with breast cancer years later have significantly elevated anti-HMdU autoantibodies (84). As Fig. 18 shows, the levels of anti-HMdU autoantibodies are remarkably stable over a period of years. The presence of high anti-HMdU autoantibody levels attests to the prooxidant conditions that have led to oxidation of bases in cellular DNA and have evoked an autoimmune response. These results also suggest that the oxidative DNA base damage (HMdU) and the biologic responses it evokes (anti-HMdU autoantibodies) start occurring early in the carcinogenic process. Such a conclusion is strengthened by data obtained by other investigators, who showed that oxidative DNA base damage is evident in DNA of individuals at risk for hormone-dependent cancers (81,84,118).

Effect of Estrogens on Oncogenes and Transcription Factors

E_2 was shown to induce macrophages to produce immediate early genes c-fos and c-jun, and the AP-1 transcription factor, which is a heterodimer of these two genes (71). AP-1-binding

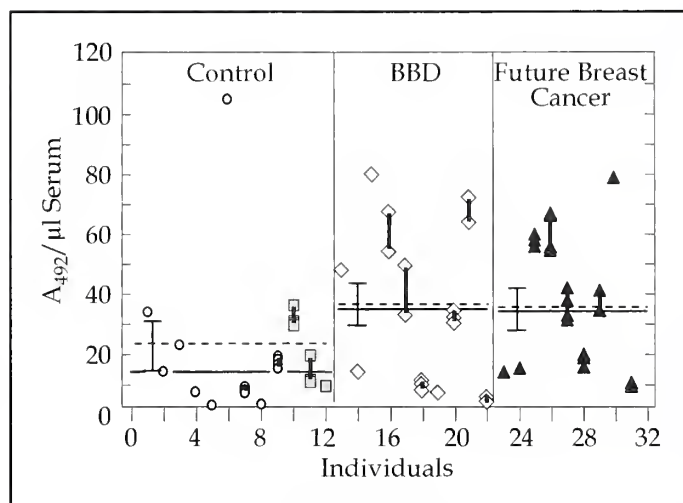


Fig. 18. Histogram of Anti-HMdU autoantibody titers in human sera (healthy controls, those with benign breast diseases [BBD], and those who were apparently healthy at the time of blood donation but were diagnosed with breast cancer 0.5–6 years later). Adapted from (84).

sites are present in promoter regions of many growth factors as well as antioxidant enzymes (112,119,120). E_2 also induces c-myc, an oncogene known to be important in tumor promotional processes, and bcl-2, a gene inhibiting apoptosis. Oxidants are known to induce all of these oncogenes, whereas antioxidants counteract their formation (57). Thus, E_2 through its ROS-inducing capabilities affects processes leading to mutations, governing tumor growth, and tumor surveillance.

Effects of estrogens on oxidant formation, oxidative DNA damage, and other cellular processes, which contribute to the carcinogenic properties of estrogens, are summarized in Table 6. Most of these effects are exactly the same as those induced by other chemical carcinogens.

Tamoxifen as an Anticarcinogen

Tamoxifen has been used as a drug that effectively prevents recurrence of breast cancer. It has also been recently shown to

Table 6. Selected properties of estrogens

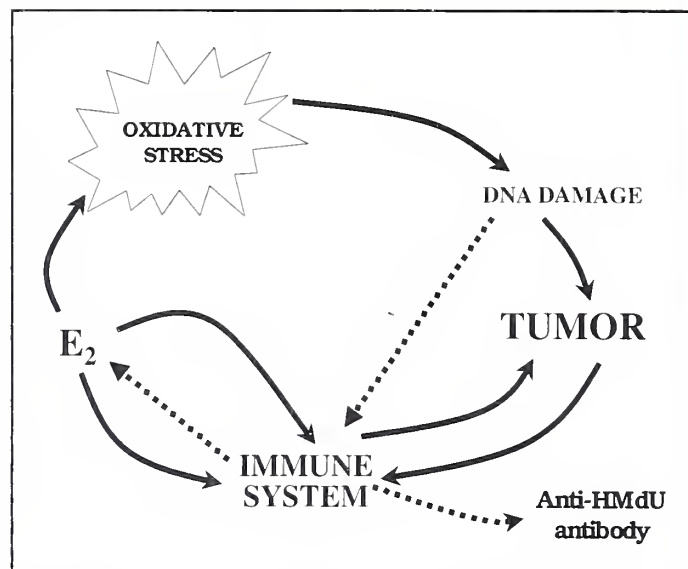
Carcinogenesis in susceptible organs	
Oxidative estrogen metabolism	
Oxidant formation and lipid peroxidation	
Genetic damage	Genetic factors
<ul style="list-style-type: none"> • DNA adducts • Oxidized DNA bases • Lipid-derived aldehyde-DNA adducts 	<ul style="list-style-type: none"> • Immediate early gene (c-fos, c-jun) • Transcription factors (AP-1) • Growth factors (TGF-β) • Oncogenes (c-myc, bcl-2)
Epithelial cell growth (\uparrow) and cell differentiation (\downarrow)	
Increase of macrophage-mediated immune responses	
<ul style="list-style-type: none"> • Proliferation • Activation • Secretions (cytokines, ROS, RNS) • Function (Antigen processing and antigen presenting to T cells) 	

decrease substantially breast cancer in women at high risk for this disease based on a strong family history. The controversy over its use as a preventive agent exists because tamoxifen increases the rate of endometrial cancer in susceptible individuals (121).

Tamoxifen exerts its antiproliferative effects on the estrogen receptor-positive cells as well as on cells lacking that receptor (122,123). This suggests that the mode of action of tamoxifen in suppressing breast cancer relies not only on its antiestrogenic properties but must involve effects on other factors important in the carcinogenic process. Tamoxifen has been shown to be a potent antitumor promoter in animal models, while estrogens can act as tumor promoters. Many of the processes known to contribute to tumor promotion are inhibited by tamoxifen (Table 7) (124). These include inhibition of hyperplasia, inflammation, ROS production, oxidative DNA base damage, and lipid peroxidation. Estrogens induce all of these processes. Hence, the fact that tamoxifen, an antibreast cancer drug, suppresses so many of the estrogen-induced prooxidant processes and factors strengthens the hypothesis of estrogen being a carcinogen that acts through elevation of oxidative stress. The interrelationships among many factors contributing to estrogen-induced oxidative stress and carcinogenesis are summarized in Fig. 19.

IS E₂ A GENOTOXIC OR EPIGENETIC CARCINOGEN?

Pharmacologic levels of estrogens are known to produce toxic effects, such as embryotoxicity, teratogenicity, and carcinogenicity (2,125,126). The molecular mechanism(s) by which estrogens cause such adverse effects are under investigation from several different angles. Recent epidemiologic and laboratory findings have increased the growing concern that instability in the genome induced by estrogens may be involved in the induction of certain types of cancer in humans (127). Estrogen-induced genotoxicity is an important contributor to the induction

**Fig. 19.** Interaction among estrogen, immune system, tumor, and oxidative stress.

of toxic effects, because estrogen receptor-mediated events by themselves cannot explain the carcinogenic and noncarcinogenic adverse properties of estrogens. The lack of mutagenic activity in bacterial and mammalian cell mutation assays (3–7) led to estrogens being categorized as nongenotoxic and nonmutagenic chemicals (8,9,128). However, more recent results, such as the arrest of DNA replication deriving from estrogen–DNA adducts, the enhancement of homologous recombination, and mutations in individual genes and in microsatellite repeat sequences clearly indicate that estrogens are able to induce multiple types of genetic insults in cells. This part of the chapter will focus on estrogen-mediated damage at the genome level leading to the development of mutations.

Indirect Evidence of Mutations Induced by Estrogens

Mutations may result from numeric changes or structural alterations in the genome. These include extra or missing copies of microsatellite DNA, transcriptional silencing, chromosomal deletions, frameshifts, amplifications, rearrangements, translocations, and other changes that interfere with the integrity of the genome. More recent experiments have shown that some estrogens are capable of producing such instability (126,129,130). For instance, estrogens induce numeric changes in chromosomes (genome mutation or aneuploidy) with and without apparent DNA damage (131). Both DES and E₂ are potent inhibitors of mitosis *in vitro* and are capable of inducing genomic mutations in cultured cells (132). Potential targets for numeric changes in chromosomes are the spindle apparatus (microtubules and centrioles), DNA, regulating proteins, and centromere. Chromosomal analysis of tissues of mice exposed perinatally to estrogen demonstrates that this treatment induces chromosomal aberrations in the same target tissues in which tumors subsequently develop (133,134). DES or E₂ treatment of hamsters produces renal chromosomal aberrations, including deletions, inversions, and translocations (135,136).

DNA damage either by free radicals or by genotoxic reactive metabolites is known to cause structural changes in chromosomes. Formation of oxygen-free radicals by redox cycling of

Table 7. Effects of tamoxifen, an antitumor promoter

Suppresses hyperplasia
Antagonizes inflammatory responses
Inhibits oxidant formation by neutrophils
Decreases oxidative DNA base damage
Decreases lipid peroxidation
Reduces MDA serum levels in breast cancer patients
Enhances apoptosis of tumor cells

estrogens or DNA modification by reactive estrogen metabolites may explain some of the structural and numeric chromosomal changes observed in response to estrogen exposure (131,132). Damage to DNA by ROS generated by estrogen treatment, i.e., 8-OHdG, lipid-DNA adducts, and DNA strand breaks, may induce structural and numeric alterations in chromosomes and may be important lesions capable of producing mutagenic changes in the genome.

Direct Evidence of Gene Mutations Induced by Estrogens

The potential of estrogens to induce mutations has been highly controversial. Previous studies of the mutagenic potential of estrogens showed that neither they nor their reactive intermediates induced mutations in the Ames bacterial reversion test or in Syrian hamster embryo cells (3–7). However, these results are not consistent with the covalent binding of reactive estrogen metabolites to bases of DNA or with the ROS-mediated changes to DNA bases, as discussed above. Both of these types of DNA lesions are capable of inducing mutations. Some earlier studies showed that DES and E_2 can increase mutations leading to ouabain resistance (137). Experiments demonstrate that DES quinone increases homologous recombination in *Escherichia coli* (138). Both DES and E_2 are mutagenic in the gpt+ Chinese hamster G12 cell line (7).

Re-examination of the mutagenic potential of E_2 at various concentrations demonstrated a weak, but detectable, mutagenicity of E_2 at the lowest dose assayed (10^{-10} μ M E_2) in V79 Chinese hamster lung cells (7; Albrecht T, Liehr JG: unpublished data). Covalent DNA adducts formed by DES quinone and CE quinone arrest the progression of cytochrome oxidase III gene synthesis (139). The mRNA for the repair enzyme DNA polymerase β obtained from DES-induced kidney tumors carries several mutations in the catalytic domain compared to that of age-matched control kidney (140). Kidney tumors and premalignant kidney of E_2 -treated hamsters contain mutations in repeat sequences of microsatellite DNA (141,142). Recently, mutational changes in an unidentified gene have been observed in the stilbene-induced hamster kidney tumor (Singh LP, Roy D: unpublished data). A high frequency of genomic rearrangements have been observed in transformed 10T1/2 mouse cell subclones treated with E_2 , indicating that this and other natural hormones may accelerate the accumulation of mutations (143).

Genetic instability manifested by somatic mutation of microsatellite repeats occurs with high frequency in clear cell adenocarcinomas of the vagina and cervix, with evidence of microsatellite instability in all DES-associated tumors examined (144). Furthermore, mutations have been reported in the p53 gene at codons 274 and 140 in hepatocellular carcinoma associated with use of oral contraceptives (145). A good association has been shown between induction of aneuploidy, DNA adducts, and estrogen-induced cell transformation (146). These findings indicate that several types of mutations induced by estrogens may not be detectable by the Salmonella reversion test or assay of gene mutations at specific narrowly defined loci in Syrian hamster embryo cells. Taken together, these findings suggest that estrogens can produce multiple types of genetic insults contributing to the induction of genomic instability. Several structural and numeric changes have already been demonstrated at the cellular level in response to DES or E_2 exposure (147).

CONCLUSIONS

In this chapter, we have outlined a large body of evidence that estrogens, including the natural hormones E_2 and E_1 , damage genetic material in various ways. Direct estrogen DNA adducts and/or indirect forms of covalent DNA alterations may be induced in experimental systems *in vitro*, cells in culture, and laboratory animals or may be detected in humans. Study of model carcinogenic PAH has led to the discovery that apurinic sites in DNA generated by depurinating adducts can lead to the mutations that trigger the cancer process. The CE metabolites, when oxidized to the electrophilic CE-Q, may react with DNA to form stable and depurinating adducts. The 4-CE that form predominantly depurinating adducts are carcinogenic, whereas the noncarcinogenic 2-CE exclusively form stable DNA adducts. Oxidation of CE also leads to overwhelming amounts of ROS that generate extensive DNA damage and contribute to tumor promotion.

Preliminary data also suggest that estrogens, including the natural hormones E_2 and E_1 , may induce gene mutations. The more recent gene mutation experiments reported in this chapter are compatible with the lack of mutagenic activity of synthetic and steroidal estrogens and their metabolites reported previously (3–7). Mutagenicity assays are designed to detect only the relatively high-mutation frequencies of potent carcinogenic compounds. In contrast, estrogens may be only weakly mutagenic, as is to be expected for endogenous compounds. Thus, mutagenicity assay conditions may have to be redesigned to detect low mutation frequencies at multiple gene loci with high accuracy and precision.

The current data presented in this chapter lead to the conclusion that estrogens are genotoxic carcinogens. Oxidation of E_1 and E_2 to CE, with the 4-CE playing the major role, is the critical pathway of activation to form ultimate electrophilic metabolites and ROS.

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Chapter 5: Tissue-Specific Synthesis and Oxidative Metabolism of Estrogens

Colin R. Jefcoate, Joachim G. Liehr, Richard J. Santen, Thomas R. Sutter, James D. Yager, Wei Yue, Steven J. Santner, Rajeshwar Tekmal, Laurence Demers, Robert Pauley, Frederick Naftolin, Gil Mor, Lev Bernstein

Estrogen exposure represents the major known risk factor for development of breast cancer in women and is implicated in the development of prostate cancer in men. Human breast tissue has been shown to be a site of oxidative metabolism of estrogen due to the presence of specific cytochrome P450 enzymes. The oxidative metabolism of 17β -estradiol (E_2) to E_2 -3,4-quinone metabolites by an E_2 -4-hydroxylase in breast tissue provides a rational hypothesis to explain the mammary carcinogenic effects of estrogen in women because this metabolite is directly genotoxic and can undergo redox cycling to form genotoxic reactive oxygen species. In this chapter, evidence in support of this hypothesis and of the role of P4501B1 as the 4-hydroxylase expressed in human breast tissue is reviewed. However, the plausibility of this hypothesis has been questioned on the grounds that insufficient E_2 is present in breast tissue to be converted to biologically significant amounts of metabolite. This critique is based on the assumption that plasma and tissue E_2 levels are concordant. However, breast cancer tissue E_2 levels are 10-fold to 50-fold higher in postmenopausal women than predicted from plasma levels. Consequently, factors must be present to alter breast tissue E_2 levels independently of plasma concentrations. One such factor may be the local production of E_2 in breast tissue through the enzyme aromatase, and the evidence supporting the expression of aromatase in breast tissue is also reviewed in this chapter. If correct, mutations or environmental factors enhancing aromatase activity might result in high tissue concentrations of E_2 that would likely be sufficient to serve as substrates for CYP1B1, given its high affinity for E_2 . This concept, if verified experimentally, would provide plausibility to the hypothesis that sufficient E_2 may be present in tissue for formation of catechol metabolites that are estrogenic and which, upon further oxidative metabolism, form genotoxic species at levels that may contribute to estrogen carcinogenesis. [J Natl Cancer Inst Monogr 2000;27:95–112]

The major known risk factors for development of breast cancer in women are associated with prolonged exposure to increased levels of estrogen. Estrogen and testosterone are also thought to be involved in the development of prostate cancer in men (see Chapter 2). From information presented in Chapters 3 and 4, it is clear that evidence is building for a role of oxidative metabolites of 17β -estradiol (E_2) and/or estrone (E_1), particularly the catechols, in breast cancer. New information implicates the catechols as signaling molecules with relative binding affinities for the human estrogen receptor that are equal to or greater than E_2 itself (1). It is also clear that, upon further oxidative metabolism, the catechol metabolites can form quinones that can directly form adducts with glutathione and guanine and adenine

bases in DNA (see Chapter 4). In particular, the 3,4-quinone forms a depurinating adduct with guanine and adenine, leaving an abasic site with mutagenic potential. In addition, as will be discussed in more detail below, the catechols are capable of redox cycling, a process accompanied by the generation of reactive oxygen species able to cause oxidative damage to lipids, proteins, and DNA.

A critical issue in relation to estrogen and the potential contribution of the catechol estrogen (CE) metabolites to breast cancer is their source. Estrogens themselves and their oxidative metabolites are formed by the activities of various cytochromes P450 (CYPs). These enzymes have dual functions, the biosynthesis and/or inactivation of physiologic regulators on the one hand and the metabolism of environmental chemicals on the other. Natural processes in which they participate include the synthesis of estrogen from cholesterol, which involves multiple, very specific CYP enzymes, typically compartmentalized into different organelles, cells, or organs. The final process in the synthesis of estrogens involves a three-step oxygenation reaction catalyzed by a single P450, CYP19 (aromatase), which converts an androgen (testosterone or androstenedione) to an estrogen (E_2 or E_1 , respectively). Numerous low-specificity CYPs are involved in the oxidative inactivation and clearance of these same steroids, drugs, and environmental pollutants. The low specificity of these P450 cytochromes, which are most abundant in liver but are also found in most cells, results in the conversion of these chemicals to multiple products with increased hydrophilicity and functional groups for subsequent metabolism. For some chemicals, particularly those that contain olefinic double bonds or aromatic rings, these products may include chemically reactive metabolites that can cause DNA damage and thereby cause errors during replication, which result in mutations. The metabolism of steroids, in conjunction with conjugation reactions (sulfation, glucuronidation, and methylation), may contribute to lowering serum and cellular levels of steroidal parent hormones

Affiliations of authors: C. R. Jefcoate, Department of Pharmacology, University of Wisconsin—Madison; J. G. Liehr, Stehlin Foundation for Cancer Research, Houston, TX; R. J. Santen, W. Yue, Division of Hematology, Oncology, and Endocrinology, Cancer Center, University of Virginia Health Science Center, Charlottesville; T. R. Sutter, W. Harry Feinstone Center for Genomic Research, University of Memphis, TN; J. D. Yager, Division of Toxicological Sciences, Department of Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD; S. J. Santner; R. Pauley, Wayne State University, Detroit, MI; R. Tekmal; Emory University, Atlanta, GA; L. Demers, Pennsylvania State University, Hershey; F. Naftolin, G. Mor, Yale University, New Haven, CT; L. Bernstein, Petrov Institute, St. Petersburg, Russia.

Correspondence to: James D. Yager, Ph.D., Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, 615 North Wolfe St., Baltimore, MD 21205 (e-mail: jyager@jhsph.edu).

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and, hence, to altered regulation of biologic activity. Identification of any new P450 cytochrome raises the question of which of these functions underlie its pattern of expression. It is most important to appreciate that the various P450 enzymes show tissue-specific expression; i.e., forms expressed in liver may not reflect those expressed in breast or prostate tissue. The implications of this are potentially enormous, since metabolites (whether of exogenous or endogenous compounds) will show tissue specificity.

The mammary gland is involved in steroid synthesis (certainly aromatase and possibly other steps), steroid metabolism (hydroxylation and conjugation), and xenobiotic metabolism (lipophilic molecules stored in fat and excreted in the milk). As will be discussed in detail below, an investigation (2) indicated that substantial levels of estrogen arise from aromatase activity localized in breast tissue. This is present in both breast epithelia and fibroblasts. Thus, estrogen biosynthesis occurs in the target tissue. Furthermore, the estrogen oxidative metabolites are formed by various CYPs. Human breast and breast tumor tissue express various CYPs. Among these is CYP1B1. As will be discussed in detail below, CYP1B1 is a catalytically efficient estrogen 4-hydroxylase. CYP1B1 is present in fibroblasts, as well as in epithelial cells (3–6); thus, it is co-localized with aromatase, the enzyme producing estrogens. This co-localization of CYP1B1 and aromatase means that the effective concentration of the 4-hydroxylated product is much higher than that indicated by the circulating levels. Human breast fibroblasts contain estrogen receptors that regulate cell growth *in vitro*, but estrogen receptors are present in only 5%–10% of the epithelial cells. A study (7) indicated that aromatase is elevated by prostaglandin E₂ (via cyclic adenosine monophosphate [cAMP]) and by various cytokines. However, very little is known about the regulation of CYP1B1 and estrogen receptors in these cells. Expression of each of these genes is also dependent on the extracellular matrix and growth factor milieu that surrounds these cells. Several presentations in this monograph raise the issue of oxidative stress (*see* Chapters 3 and 4), mediated by the 4-CE/*o*-quinone redox cycling. One possibility is that this may also contribute to signaling that promotes aromatase expression. The remainder of this chapter will provide greater detail regarding the expression and potential roles of aromatase, CYP1B1, and CE, particularly 4-hydroxy-E₂ (4-OHE₂), in breast cancer.

POTENTIAL ROLE OF AROMATASE OVEREXPRESSION IN THE GENESIS OF BREAST CARCINOMA

Estrogens and Risk of Breast Cancer

Evidence from multiple sources suggests that estrogens are involved in the genesis of breast carcinoma. Administration of exogenous estrogens causes breast cancer in rodents (8). Breast tumors induced by the potent carcinogen 7,12-dimethylbenz[*a*]anthracene can be delayed or prevented by castration and administration of anti-estrogens (9). Aromatase inhibitors prevent the spontaneous development of breast tumors in aging Sprague-Dawley rats (10). By the age of 2 years, approximately 50% of these animals develop either benign or malignant breast lesions (Fig. 1) (10). Increasing doses of the aromatase inhibitor fadrozole inhibit these tumors in a dose-dependent fashion. Notably, 1.25 mg of fadrozole/kg of body weight daily causes 100% inhibition. Taken together, these data provide strong evidence that estrogens are involved in the carcinogenic process, resulting in breast neoplasms in animals.

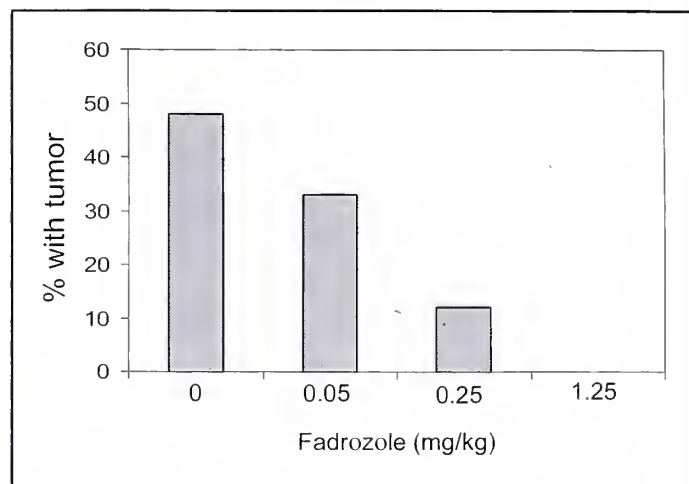


Fig. 1. Inhibition of spontaneous benign and malignant breast tumors in aging Sprague-Dawley rats with administration of the aromatase inhibitor fadrozole. No tumors appeared in animals receiving the 1.25 mg/kg dose. Figure adapted from the data of Gunson et al. (10).

Indirect evidence in women also supports a role for estrogen in the genesis of breast cancer. Early menarche and late menopause are associated with an increased risk of breast cancer. These factors result in prolonged exposure of the breast to estrogen. Prospective epidemiologic studies (11–14) detected an increase in the plasma levels of E₂ in women developing breast cancer 5 or more years later. Obesity is also associated with a greater risk of breast cancer (15). This relationship might also be explained by increased estrogen production, since the degree of obesity correlates linearly with total-body aromatase activity (16). Aromatase catalyzes the rate-limiting step in estrogen biosynthesis, the conversion of androgens to estrogens.

Additional indirect evidence regarding estrogens and breast cancer derives from analyses of the rate of breast cancer in women receiving estrogen replacement therapy during the menopause. More than 50 studies are now available to examine this relationship [reviewed in (17)]. Six meta-analyses have pooled this information, and a critical review of these data allows several points to be made. The first is that no randomized, controlled, double-blind studies have been conducted to demonstrate conclusively that estrogen replacement therapy during the menopause increases the risk of breast cancer. Only observational studies are available. Secondly, while susceptible to several biases, most of these studies show an increased risk of breast cancer with the use of estrogen replacement therapy for a period of more than 5–10 years. The relative risk of breast cancer under these circumstances increases by about 30%. Thirdly, the absolute increased risk is small, approximating one additional breast cancer case in 100 women of age 50 years who have taken estrogen for at least 10 years.

The conclusion that these data prove that estrogen replacement therapy is associated with an increased risk of breast cancer is controversial. However, as current studies evolve, the evidence increases. For example, the Nurses' Health Study (18) now involves more than 725 550 patient-years of observation over a 10-year period. In this study, the risk of breast cancer increased with more than 5 years of current estrogen use in women of all ages over 50 years. This cohort study allows adjustment for most, but not all, confounding factors and provides the most convincing evidence of a relationship between estrogen

ingestion and breast cancer. The definitive study, conducted as part of the Women's Health Initiative, involves a randomized, placebo-controlled trial. The results of this study will not be available until midway into the first decade of the 21st century.

Correlations between estrogen levels and subsequent risk of breast cancer have not, until recently, been positive. Earlier studies measured levels of urinary estrogens, plasma estrogens, or the free fraction of E₂ with insensitive or relatively nonspecific methods. Conflicting results were reported, and the common view was that estrogens were not elevated in women who would later develop breast cancer (19,20). Recently, however, Toniolo et al. (11) and three other groups (12–14) demonstrated increased levels of E₂ and its precursor, testosterone, in women found to develop breast cancer prospectively 5 or more years later. While not every group could replicate these results (19,20), the majority of data support such a relationship.

Further evidence regarding estrogen production and breast cancer risk has been provided by experiments demonstrating that a reduction in estrogen production in women reduces the incidence of breast cancer (21,22). Data from two classic studies (21,22) are consistent with such an effect (Fig. 2). One of these studies (22) examined the incidence of breast cancer in a group of women who had undergone bilateral oophorectomy before

age 35 years. The control group consisted of women subjected to a unilateral oophorectomy at the same ages. The end point of the study was the ratio between observed and expected breast cancers in these two groups of women. After a period of 20 years, the women undergoing bilateral oophorectomy had a 75% reduction in the incidence of breast carcinoma (Fig. 2, A). In the other study (21), with a similar design, the decrease in breast cancer incidence over that expected gradually declined as a function of time after oophorectomy (Fig. 2, B). Although these studies were also subject to bias, they provide compelling evidence that ovarian factors, and presumably E₂, are involved in the genesis of breast carcinoma.

Sources of Estrogen

The sources of E₂ production in women are important to consider, since overproduction may result from altered regulation at any site. Estrogen can be made in several tissues. Aromatase, the enzyme catalyzing the rate-limiting step in estrogen biosynthesis, is widely present throughout the body. The premenopausal ovary, which contains the highest level of aromatase, except for the placenta, is the major source of E₂ during the premenopausal years. Peripheral adipose tissue also contains aromatase and is a major source of this enzyme, since the mass of adipose tissue (particularly in obese women) is substantial. Breast tissue itself contains aromatase, both in its fatty components and in its epithelial cells, and can synthesize estrogen *in situ*.

Importance of *In Situ* Aromatase in Breast Tissue

Emerging evidence suggests that estrogen produced *in situ*, as opposed to E₂ made in other tissues and delivered to the breast via an endocrine mechanism, plays a major biologic role in breast physiology. Several lines of evidence support this concept. Demonstration of the aromatase enzyme and its messenger RNA in breast tissue by immunohistochemical and molecular biologic techniques, studies in nude mice to show that the amount of estrogen made locally causes biologic effects, and clinical studies of aromatase inhibitors in patients provide proof of the importance of *in situ* production of estrogen in breast tissue.

Several groups of investigators (23–25) over the past few years have demonstrated the aromatase enzyme in breast tissue. Both radiometric and product isolation methods demonstrated that tritiated androgens could be converted to estrogens in human breast cancer tissue as evidence of aromatase activity. The biologic significance of this finding was initially questioned, since the amount of enzyme present, compared with that in placenta or ovary, was low (26). In this regard, other investigators postulated that focal concentrations of aromatase in selected cell populations might be high, but overall activity in breast cancer tissue might be low. This might occur because of the presence of fibrous or other tissues in the tumor, which would dilute the concentration of enzyme in the tumor overall. To examine this possibility, immunohistochemical studies were used to detect aromatase in individual breast cancer cells. Resulting data (27–29) demonstrated high levels of aromatase staining in individual cells, supporting the concept that aromatase might act in an autocrine or paracrine fashion in breast tissue (Fig. 3).

Controversy exists at present whether aromatase activity is

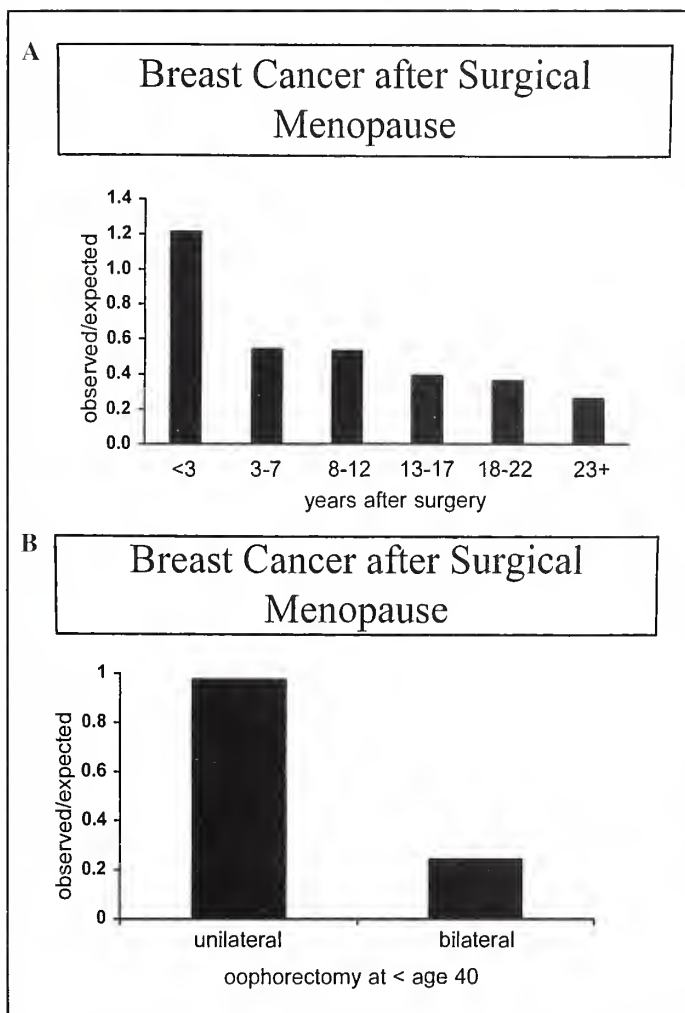


Fig. 2. A) Observed over expected rates of breast cancer over time after surgical menopause. Adapted from the data of Trichopoulos et al. (21). B) Observed over expected rates of breast cancer in women undergoing unilateral and bilateral oophorectomy. Adapted from the data of Feinleib et al. (22).

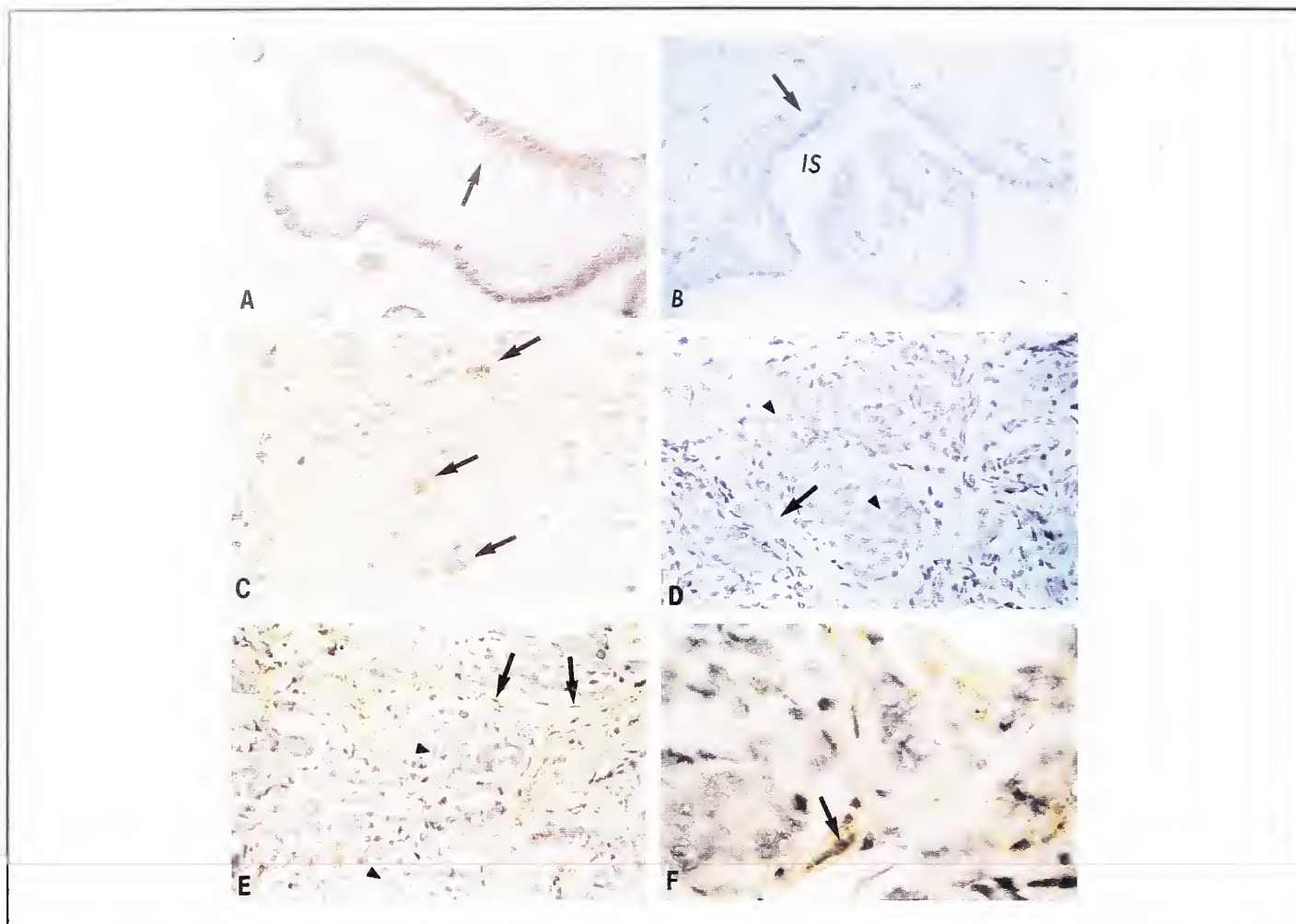


Fig. 3. Immunohistochemical staining for aromatase in human breast cancer. **A)** Section of human placental villus stained with the Harada antibody. **B)** Control section of human placenta stained with an irrelevant antibody, anti-neuropeptide Y. **C)** Section of human breast tumor stained with the anti-aromatase antibody. **D)** Control section of human breast tumor stained with an irrelevant antibody. **E)** Section of human breast tumor stained with the anti-aromatase antibody showing stromal spindle cells positively stained. **F)** Photomicrograph of greater magnification from the periphery of a group of tumor epithelial cells showing positive staining of stromal spindle cells. Reprinted from (27) with the permission of authors and publisher.

predominantly in epithelial cancer cells or in the surrounding stromal cells (30,31). Certain monoclonal antibodies, used in conjunction with antigen retrieval techniques, suggest that the majority of aromatase is in epithelial cells (30). Monospecific polyclonal antibodies, utilized on the same tissue sections, show a preponderance of aromatase activity in stromal cells (27,32,33). To provide additional data regarding stromal aromatase, Santen and his group (33) grew stromal cells isolated from breast cancer lesions as well as from benign tissue surrounding the tumors. They found that isolated stromal cells from breast cancer tissue contain high levels of aromatase enzyme when stimulated by dexamethasone, phorbol esters, and cAMP in combination. Aromatase enzyme activity, assessed by a radiometric assay, increases by nearly four logs in response to this combination of enhancers, and message increases 30-fold (Fig. 4). Other investigators have found that aromatase message levels are higher in areas of breast cancer with high stromal cell content than in areas with low content. Quantitative assessment using a histologic scoring system and immunohistochemistry detected average H-scores of 13 (range, 0–45) for stromal cells and 4 (range, 1.4–16) for stroma in 26 human breast tumors (27). This technique takes into account the relative abundance of stromal

and epithelial cells in the tumor (27). Considered together, these data support the biologic importance of aromatase in breast cancer tissue and suggest that stroma may contribute to a greater extent to this process than epithelial cells.

Further support for the importance of aromatase in breast tissue itself derives from studies in a nude mouse model developed by Yue et al. (34). Using this model, these investigators (34) examined the relative importance of uptake from plasma versus local E_2 synthesis in breast tissue. This model involves the use of MCF-7 breast cancer cells transfected stably with aromatase (A+) that are implanted on one side of castrated nude mice. On the other side, sham-transfected MCF-7 cells (A–) are implanted. Administration of the aromatase substrate androstenedione causes no growth stimulation of aromatase-negative cells (Fig. 5). This important control demonstrates that no aromatase activity is present in non-breast tissue in the mouse. Aromatase-positive cells implanted on the other side of the same animals are stimulated to grow by androstenedione, providing evidence of the biologic effect of aromatase present locally in the breast. The aromatase inhibitor 4-hydroxyandrostenedione blocked this growth effect. One can conclude that the growth stimulation cannot have occurred as a result of peripheral con-

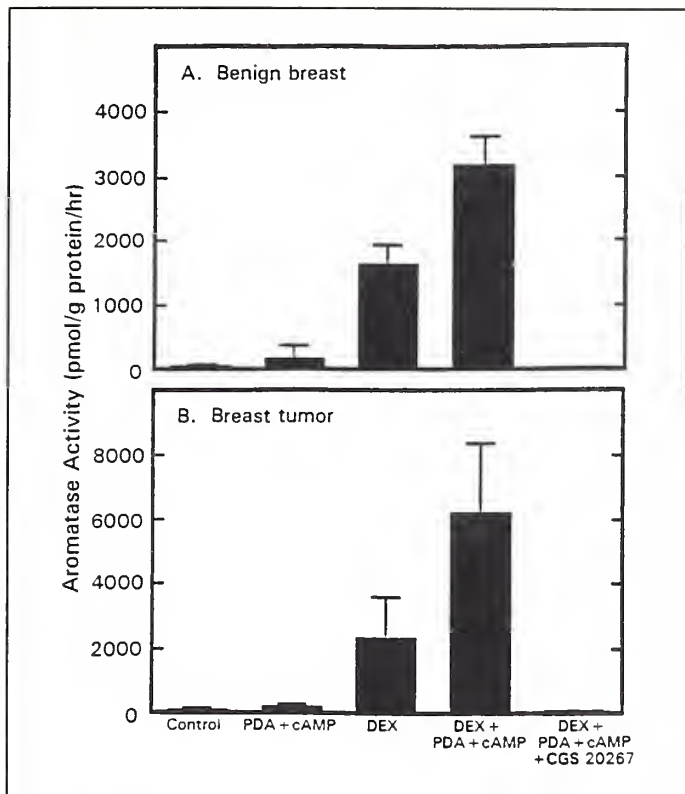


Fig. 4. Regulation of aromatase enzyme activity in benign breast and breast tumor stromal cell cultures. A) Enzyme activity in myofibroblasts isolated from human breast tissue. Basal activity was compared with phorbol ester plus cyclic adenosine monophosphate (cAMP) (100 nmol/L phorbol diacetate [PDA] and 1 nmol/L cAMP), 100 nmol/L dexamethasone (DEX) alone, the combination of DEX, PDA, and cAMP, and the addition of the aromatase inhibitor letrozole (1 μ mol/L) to the combination treatment (Ciba Geigy Substance 20267). The results of cultures from three patients were used. Data reprinted from (33) with the permission of authors and publisher.

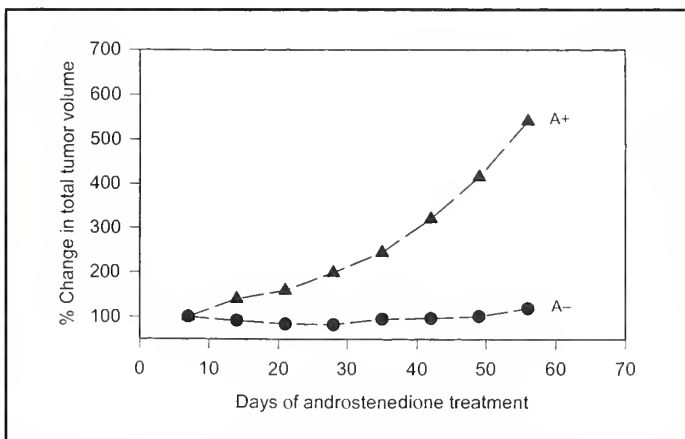


Fig. 5. Change in tumor volumes in aromatase-transfected MCF-7 cells (A+) and in sham-transfected cells (A-). Data reprinted from (34) with the permission of authors and publisher.

version to E_2 , which would have stimulated the aromatase-negative cells as well. As expected, the levels of E_2 in aromatase-positive tumors markedly exceeded those in aromatase-negative tumors (Fig. 6).

The relative importance of *in situ* production versus uptake of E_2 from plasma was then examined. Silastic implants designed

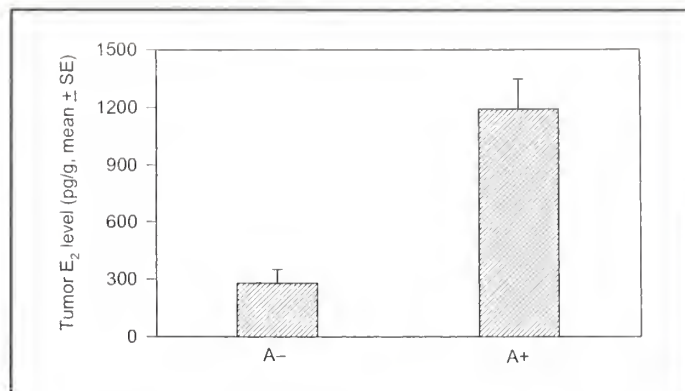


Fig. 6. Tumor estradiol (E_2) concentrations in aromatase-transfected (A+) and sham-transfected cells (A-) implanted into and grown in nude mice. Data reprinted from (34) with the permission of authors and publisher. SE = standard error.

to produce plasma estrogen levels ranging from 5 to 20 pg/mL were implanted into castrate animals to evaluate the effect of E_2 uptake. Androstenedione was administered to others to examine *in situ* production. With this experimental system, tissue E_2 levels and tumor growth were higher as a result of *in situ* aromatization than from plasma delivery of estrogen [data shown in (34)].

This series of experiments in mice supports the hypothesis that an important determinant of tissue E_2 levels is local production in the breast. If this hypothesis is correct, the level of E_2 produced in breast tissue may be the most important determinant of E_2 -induced carcinogenesis. This conclusion is supported by the direct measurement of aromatase activity with elegant isotopic techniques in human breast tumors by Reed et al. (35). These investigators demonstrated that $83\% \pm 9\%$ (standard deviation) of tumor estrogen levels resulted from *in situ* aromatase in four of six tumors. In the other two tumors, no estrogen synthesis in the breast itself could be demonstrated. Although speculative, the hypothesis of *in situ* E_2 synthesis could explain the relatively poor correlations between use of estrogens for menopausal replacement therapy and breast cancer risk. This might also explain why it has been difficult to demonstrate higher estrogen levels in women who will later develop breast cancer. If the local synthesis hypothesis is correct, measurement of the concentration of E_2 in breast tissue itself would be the most precise predictor of later development of breast cancer. This concept is supported by the fact that plasma and tissue E_2 levels are not well correlated. The ratio of tissue-to-plasma E_2 levels in premenopausal women approximates 1:1, whereas in postmenopausal women the ratio is 10–50:1 (36). Taken together, these data suggest that certain factors present in breast tissue can influence local production of estrogen and that these may be the prime determinants of tissue estrogen concentrations. If these concepts are correct, elevated plasma levels of estrogen would be associated with high tissue concentrations in some, but not in all, patients and breast cancer risk might only be increased in those with high tissue levels.

Mechanism of Carcinogenesis

After considering the sources of E_2 available for stimulating breast tissue, one must consider how E_2 causes breast cancer. The predominant theory at the present time relates to effects of estrogen on cell growth (37). Enhanced cell proliferation, in-

duced either by endogenous or by exogenous estrogens, increases the number of cell divisions and, by inference, the proportionate number of mutations. With an enhanced rate of proliferation, the time available for DNA repair is reduced. In addition, single-stranded DNA, present during cell division, is more susceptible to damage than double-stranded DNA, and gene duplication can occur.

Another current theory, discussed in Chapters 3 and 4 and in the following section, is that estrogens can be metabolized to genotoxic products. These two current theories of enhanced cell proliferation and genotoxic metabolites are not mutually exclusive but could act in an additive or even synergistic fashion. For example, DNA damage originating from CEs would be propagated more rapidly by increased cellular proliferation, and insufficient time might be available for DNA repair. Additional data will be required to determine the precise interactions between these two pathways of carcinogenesis.

The major critique of the genotoxic metabolite theory is that estrogen levels are not sufficiently high to produce biologically relevant amounts of these metabolites. This critique, however, is based on an analysis of plasma E_2 levels and not tissue levels. If E_2 is synthesized locally in breast tissue, the levels would be higher than expected from plasma concentrations. This concept is supported by the fact that E_2 concentrations in breast cancers from postmenopausal women are as high as those from premenopausal women. This is surprising, since the levels of E_2 in the plasma of premenopausal women are 10- to 50-fold higher than those in the plasma of postmenopausal women (36).

Hypothesis of Aromatase Overexpression

As a means of integrating these data, Santen et al. (personal communication) have postulated that aromatase overexpression in breast tissue may be a cause of breast cancer. Through aromatase overexpression, tissue levels of E_2 would be sufficiently high to undergo metabolism to biologically important quantities of genotoxic metabolites. Four separate models of aromatase overexpression and breast cancer have been well characterized and provide strong support for this hypothesis. Three involve the hyperplastic alveolar nodule (HAN) model systems. Zhang and Medina (38) have developed a series of transplantable breast explants that grow in the mammary fat pads of highly inbred strains of mice. Two of these are induced by carcinogens and are called the C4 and C5 HAN. One is induced by hormonal stimulation and is called the D2 HAN. Upon serial passage in mammary fat pads, these lesions develop frank cancer with an incidence that approaches 90% under certain conditions. Each of these HAN models has been shown to have an insertional mutation called Int 5. This mutation has now been characterized and involves the insertion of a portion of the long terminal repeat of the mouse mammary tumor virus into genomic DNA (39-41). Of great interest is the fact that, in each of these models, the insertion is into the 3' untranslated region of the tenth exon of the aromatase gene. This results in overexpression of the aromatase gene and, by inference, in tumor development. The fourth model is a transgenic mouse model in which aromatase is overexpressed, predominantly in mammary tissue (42). These animals develop atypical ductal hyperplasia, a type of lesion that predicts an increased rate of breast cancer development when found in patients. They also develop fibroadenomas, typical ductal hyperplasia, and dysplasia, lesions that are not associated with an increased risk of breast cancer in women. Rarely do the aromatase-transfected animals develop frank breast cancer.

Evidence of Aromatase in Benign Breast Tissue

Several laboratories have obtained evidence that malignant breast tissue contains both aromatase message and enzyme activity. However, if aromatase overexpression is important in the genesis of breast cancer, this enzyme must be present in benign breast tissue as well. To assess this possibility, core and excisional biopsy specimens containing atypical ductal hyperplasia were evaluated with an immunohistochemical method using a monospecific polyclonal antibody (43). This technique revealed aromatase immunohistochemical staining in both stromal and epithelial cells contained in the hyperplastic lesions. In the surrounding normal tissue, aromatase staining was present predominantly in glandular epithelial cells but to a lesser extent in stroma.

Surprisingly, macrophages with substantial aromatase activity were also detected (43). This finding led to an extensive series of experiments to verify that macrophages indeed express aromatase. THP-1 cells, a malignant cell line that can be differentiated into macrophages upon exposure to phorbol esters (44), were used. These cells contained aromatase enzyme activity with levels close to those found in human placenta. The aromatase inhibitor letrozole completely inhibited this activity. Conditioned media from these cells, exposed to the aromatase substrate testosterone, stimulated the growth of E_2 -responsive MCF-7 indicator cells. As evidence of specificity, growth of indicator cells could be blocked with letrozole or with the pure anti-estrogen ICI 182780.

As further evidence of aromatase expression in macrophages, human monocytes were examined, basally and after differentiation into macrophages, in tissue culture with the addition of phorbol esters. These cells contained aromatase message when differentiated into macrophages but not under control conditions. Finally, monoclonal antibodies specific for macrophages were used to demonstrate, by double labeling, that the cells in the breast that contained aromatase were in fact macrophages.

The production of E_2 by macrophages is of further interest because chemokines regulating tissue invasion by macrophages are also controlled by estrogen. The levels of macrophage chemokine 1 (MCP-1) are lowered by E_2 in MCF-7 breast cancer cells and in other tissues (see Chapter 8). It is interesting that a classical negative feedback loop could exist whereby E_2 lowers MCP-1, which would result in a decrease in invasion of tissue by macrophages. This would result in a lower production of E_2 in that tissue. As a consequence, the levels of MCP-1 would increase and the macrophages would again be stimulated to invade the tissue. Since breast tumors contain a substantial number of macrophages, their contribution to local E_2 production could be biologically important.

These data suggest that breast tissue can make E_2 from epithelial cells, from stroma, and from macrophages that infiltrate normal tissue. Potentially, either one of these three cell types could overexpress aromatase and provide sufficient amounts of E_2 locally to allow conversion to genotoxic quinone metabolites. Several examples of aromatase overexpression are known to exist. A rat Leydig cell tumor overexpresses aromatase through activation of a cAMP-dependent enhancer of aromatase (45). The breast tissue of goats is the major source of aromatase prior to parturition, and bilateral mastectomy delays the time of parturition (46). The Sebright bantam syndrome is caused by aromatase overexpression, which feminizes the feather pattern of roosters and gives them the phenotypic appearance of chickens

(47). Familial causes of aromatase overexpression occur in patients, resulting in prepubertal gynecomastia in boys and precocious thelarche and/or macromastia in girls. The Peutz-Jeghers syndrome is characterized by aromatase overproduction and leads to testicular tumors in boys and ovarian tumors in girls. Each of these examples provides evidence that aromatase overexpression is somewhat common in animals and in patients (48).

Mechanism of Aromatase Overexpression

A variety of potential mechanisms could result in aromatase overexpression. Aromatase transcription is regulated by multiple enhancers, including cAMP, phorbol esters, dexamethasone, prostaglandin E_2 , transforming growth factor- β and interferon gamma among others (49). Fibroblasts isolated from breast tumors as well as from benign tissue surrounding the tumors contain aromatase. The activity of this enzyme and its message can be stimulated up to 10000-fold in cell culture with addition of phorbol esters, cAMP, and dexamethasone (Fig. 4) (41). Activating mutations involving any of these or other steps could result in aromatase overexpression in breast tissue. Simpson and co-workers (50) have postulated that prostaglandin E_2 may be important in this process and have pointed out that use of non-steroidal anti-inflammatory agents is associated with a decreased incidence of breast cancer in women. These agents are known to block prostaglandin E_2 production and putatively could decrease breast cancer through this mechanism.

Prevention of Breast Cancer

If aromatase overexpression were an etiologic factor for breast cancer, third-generation aromatase inhibitors might be used for prevention. In premenopausal women, it would be possible to block estrogen production in breast tissue without affecting ovarian E_2 synthesis. The ovary is relatively resistant to aromatase inhibitors because of the extremely high levels of androstenedione present as substrate in the ovary (51). While these concepts are speculative, further evaluation of aromatase expression in various premalignant breast lesions is warranted.

METABOLIC ACTIVATION OF ESTROGENS BY 4-HYDROXYLATION

Role of Metabolism in Estrogen-Induced Cancer

The oxidative metabolism of estrogens has been studied in detail as part of investigations of the regulation and control of hormonal action and has been reviewed by Zhu and Conney (52). In this section, estrogen metabolism will be discussed only to the extent that it affects induction of tumors by this hormone. The metabolic activation of estrogens has increasingly been suspected to play a role in the carcinogenic process (53–55) because the modulation of metabolic oxidation affects E_2 -induced tumor incidence in a rodent model, the kidney tumor in male Syrian hamsters, in a way that is not consistent with previous hypotheses. Estrogens have previously been postulated to act in hormone-associated cancers, including breast cancer, primarily by estrogen receptor-mediated proliferation of cells mutated by spontaneous replication errors [reviewed by Feigelson and Henderson (56)]. According to this hypothesis, inhibition of estrogen metabolism should enhance tumor formation, since estrogen metabolites are less hormonally active than the parent E_2 or E_1 . In contrast, α -naphthoflavone, an inhibitor of estrogen hydroxylation (57,58), and ascorbic acid (vitamin C), a reducing agent

known to reduce estrogen quinone intermediates to their hydroquinones (59), either completely or partially inhibited kidney tumor induction in hamsters by E_2 (Table 1) (59–61). Neither of these chemicals is known to have estrogen agonist or antagonist activities and, thus, could not have interfered with the estrogen receptor-mediated proliferation of mutated cells. In addition, 17 α -ethinylestradiol, a poor carcinogen in the hamster kidney tumor model (62), is converted to catechol metabolites at much lower rates than E_2 (63). All of these data are consistent with the conclusion that metabolic activation of estrogens is necessary for the induction of tumors by estrogenic hormones (53–55). This metabolic activation of estrogens, as a necessary part of the tumorigenesis process, has been proposed by analogy to metabolic activation of carcinogenic chemicals such as benzo[a]pyrene or other carcinogenic hydrocarbons (64). Moreover, the conversion to catechol metabolites and their further metabolism to quinone and semiquinone intermediates were explored, since such metabolites have been shown to play a role in DNA damage induced by other carcinogens (65).

Formation of CEs

CEs, 2- or 4-hydroxylated E_2 or E_1 , are the focus of metabolic research in the context of estrogen-induced cancer because these compounds are major oxidized metabolites of estrogenic hormones in most mammalian species (66,67) and are precursors to reactive intermediates (53–55). For instance, catechols including CEs may be oxidized chemically or by enzymatic processes to semiquinones, which are free radicals, and further to reactive quinone intermediates (68–71). These semiquinone/quinone species react with nucleophilic sulfur- or nitrogen-rich endogenous chemicals including nucleic acids (71–74). Therefore, the formation and metabolic activation of CEs, specifically 4-hydroxylated estrogens, are described below.

Catechol Formation by Aromatic Oxidation of Estrogens

2-Hydroxylation of steroidal estrogens is the major metabolic oxidation of estrogenic hormones in most mammalian species (Fig. 7) (66,67). For instance, in human or hamster livers, the 2-hydroxylation is catalyzed by CYP 3A isoforms, whereas CYP 1A isoforms represent the predominant estrogen-2-hydroxylase activity in extrahepatic tissues (57,75–77). These estrogen-2-hydroxylases convert E_2 to approximately 80%–85% 2-hydroxyestradiol (2-OHE $_2$) and, because of a lack of specificity of the enzyme(s), 15%–20% of 4-OHE $_2$ (78), as shown, for instance, for liver in Table 2. In contrast, specific estrogen-4-hydroxylase(s) that convert E_2 mainly to 4-OHE $_2$ (3) have been identified (78–80) in those organs of rodents in which chronic estrogen exposure induces malignant or benign tumors, such as in hamster kidney (62), mouse uterus (81), or rat pituitary (82).

Table 1. Reduced incidence of estrogen-induced kidney tumors in male Syrian golden hamsters by modulation of estrogen metabolism

Treatment	Kidney tumors, % tumor-bearing animals	Reference No.
None	0	(62)
17 β -Estradiol	100	(62)
17 β -Estradiol + α -naphthoflavone	0	(61)
17 β -Estradiol + ascorbic acid (vitamin C)	50	(60)
17 α -Ethinylestradiol	10	(62)
2-Fluoroestradiol	0	(84)

Fig. 7. Formation and catabolism of catechol estrogens (CEs). Estradiol is converted to 2-hydroxyestradiol (2-OHE₂) by reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent estrogen-2-hydroxylases, which form a small amount of 4-hydroxyestradiol (4-OHE₂) by a lack of specificity of the enzymes. Conversely, NADPH-dependent estrogen-4-hydroxylases form preferentially 4-OHE₂ and a small amount of 2-OHE₂. In addition, organic hydroperoxide (OHP)-dependent estrogen-2/4-hydroxylase(s) form approximately equal amounts of both catechol products. Both 2-OHE₂ and 4-OHE₂ may be converted to the corresponding methoxyestrogens (2-OCH₃E₂ and 4-OCH₃E₂, respectively) catalyzed by catechol-*O*-methyltransferase or other conjugate metabolites catalyzed by uridine diphosphate (UDP) glucuronyl transferases or other conjugating enzymes. These phase II metabolites, in turn, may be converted back to CEs by demethylating enzyme activities, glucuronidase, or other enzyme activities.

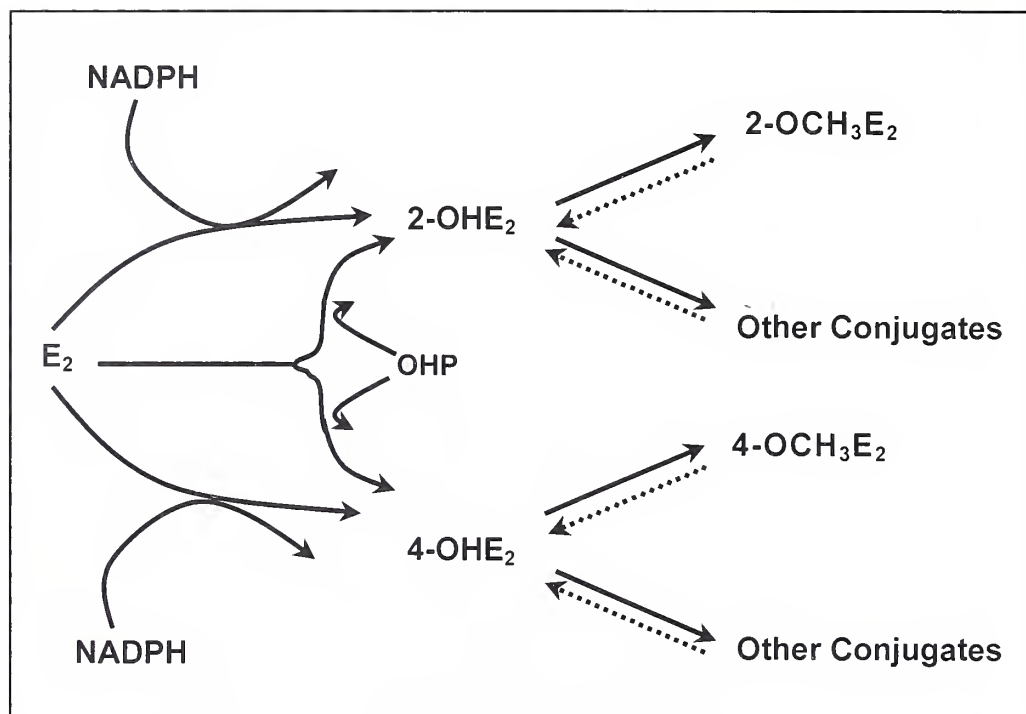


Table 2. Metabolic conversion of 10 μ M 17 β -estradiol (E₂) to catechol estrogens by microsomal preparations of target organs of estrogen-induced cancer

Tissue	2-Hydroxy-E ₂ *	4-Hydroxy-E ₂ *	$\frac{4\text{-Hydroxy-E}_2}{2\text{-Hydroxy-E}_2}$	Reference No.
Hamster liver: control	365	52.4	0.14	(78)
Hamster kidney				
Control	13.4	7.2	0.5	
E ₂ -treated	2.2	6.1	2.8	
CD-1 mouse uterus: control	0.1	1.3	13.0	(80)
Sprague-Dawley rat pituitary				
Control	0.03	0.29	9.7	(79)
E ₂ -treated	0.08	0.24	3.0	

*Values in column = picomoles per milligram protein per minute.

The specific formation of 4-hydroxylated estrogens is important because, in the hamster kidney tumor model, 4-OHE₂ is as carcinogenic as E₂, whereas 2-hydroxylated estrogens did not induce any tumors (83–85). In addition to the specific reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent estrogen 2- or 4-hydroxylation, an organic hydroperoxide-dependent estrogen 2- and 4-hydroxylase activity has been detected, which produces both catechols in roughly equal amounts (78,86).

In humans, the predominant conversion of E₂ to 4-OHE₂ has been detected in microsomes of uterine myometrium and fibroids—i.e., benign uterine myomas (87)—and in benign and malignant mammary tumors (88). In addition, a specific estrogen-4-hydroxylase activity has been identified in MCF-7 breast cancer cells, which can be induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a common environmental pollutant (89,90). As described in the introduction to this chapter, this human estrogen-4-hydroxylase activity was designated as cytochrome P4501B1 (CYP1B1), a novel extrahepatic isozyme detected in mammary tissue, ovary, adrenal gland, uterus, and elsewhere (89–91). In one reported measurement of estrogen metabolite concentrations in a human breast cancer extract (92),

the ratio of 4-OHE₂ to 2-OHE₂ was determined to be 4:1. The same 4:1 ratio was detected for the rates of formation of these CEs by breast cancer microsomes (88). It was concluded from these studies that, in rodent organs prone to estrogen-induced cancer or in human uterus or breast, which are targets of hormone-associated cancers, the predominant formation of 4-OHE₂ may result in elevated concentrations of this carcinogenic estrogen metabolite in these tissues.

Catechol Formation by Deconjugation of Conjugated Estrogens

De novo formation of CEs by hydroxylation of E₁ or E₂ by CYP enzymes (66,67) is only one of several pathways of CE formation. Other pathways include the deconjugation of conjugated estrogen metabolites. The demethylation of methoxyestrogens has been investigated with the use of liver and kidney microsomes of male Syrian hamsters (93). Rates of demethylation of 2- and 4-methoxyestradiol by kidney microsomes are comparable, whereas the rate of demethylation of 2-methoxyestradiol by liver microsomes is approximately fivefold higher than that of 4-methoxyestradiol (Table 3) (93). The rates of renal demethylation of methoxyestrogens are comparable to the rates

Table 3. Kinetic parameters for catechol estrogen formation by hamster microsomes catalyzed either by aromatic hydroxylation of 17 β -estradiol (E₂) or by demethylation of methoxyestrogens*

Product†	Aromatic hydroxylation		Demethylation of methoxyestrogens	
	K_m , μM	V_{max} , pmol/mg protein per min	K_m , μM	V_{max} , pmol/mg protein per min
Liver				
2-OHE ₂	28	1573	15.4	606
4-OHE ₂	26	453	11.5	109
Kidney				
2-OHE ₂	11.8	24.3	5.2	24
4-OHE ₂	8.7	31.9	15.5	30

*2-OHE₂ = 2-hydroxyestradiol; 4-OHE₂ = 4-hydroxyestradiol; K_m = Michaelis constant; V_{max} = maximum initial velocity of an enzyme reaction.

†Data are taken from Weisz et al. (78) and Zhu et al. (93).

of direct 2- and 4-hydroxylation of E₂ by kidney microsomes, whereas the rates of hepatic demethylation are approximately one fifth of the corresponding *de novo* hydroxylation rates (78). These data demonstrate that metabolic demethylation of methoxyestrogens is an important source of CE metabolites in the hamster kidney, a target of estrogen-induced carcinogenesis (62), whereas CE formation by direct aromatic hydroxylation of E₂ predominates in liver, where this hormone does not induce tumors under these conditions. The identities of the CYP enzymes catalyzing demethylation of methoxyestrogens are not known. CYP 3A enzymes have been identified as one of the activities capable of catalyzing the *N*-demethylation of a xenobiotic substrate (94). Increased demethylase activity for methoxyestrogens in liver microsomes from phenobarbital-treated rats also indicates participation of CYP 2B isoforms in this reaction (95). However, specific identification of the enzymes catalyzing this reaction requires further research.

Other phase II estrogen metabolites, such as E₂- and E₁-3-D-glucuronides, are also deconjugated to the parent hormones by

lysosomal glucuronidases (93). For instance, lysosomes from male Syrian hamster kidney catalyze the deconjugation of estrogen glucuronides at rates that are one-third to two-thirds greater than corresponding rates by liver lysosomes. Treatment of hamsters with E₂ implants for 9 days increases lysosomal glucuronidase activities for these estrogen glucuronides by 15%–25% in kidney and doubles the activities in liver, but it does not alter their corresponding K_m (i.e., Michaelis constant) values (93). Microsomal glucuronidase activities are approximately 10%–20% of lysosomal activities and do not appear to contribute appreciably to deconjugation of glucuronide metabolites. These results have been obtained with E₂- and E₁-3-D-glucuronides as substrates. However, CE-glucuronide metabolites exist (96) and may also be deconjugated by these enzymes. Therefore, conjugates of both CE metabolites and of parent hormones formed in the liver may circulate and be deconjugated in extrahepatic tissues, including in organs prone to hormonal cancer. For instance, comparable concentrations of 2- and 4-OHE₂ have been found in the kidney and liver of male Syrian hamsters treated with E₂, despite the fact that renal activities of estrogen-2- and 4-hydroxylases are only one-tenth to one-twentieth the hepatic values (97). These comparable estrogen concentrations suggest that, in addition to direct aromatic hydroxylation of parent hormones, other metabolic pathways of CE formation exist in the kidney, such as deconjugation of methoxyestrogens and glucuronide metabolites. In extrahepatic tissue, such as in the hamster kidney, deconjugation reactions may be as important a source of CEs as aromatic hydroxylation of parent hormone.

Metabolic Activation by Redox Cycling

CEs, including 4-OHE₂, are capable of metabolic redox cycling. This process consists of the organic hydroperoxide-dependent oxidation of the CEs (the hydroquinone) to the quinone and the NADPH-dependent CYP reductase-catalyzed reduction of the quinone intermediate back to the hydroquinone (Fig. 8) (68). The quinones may react with nucleic acids and

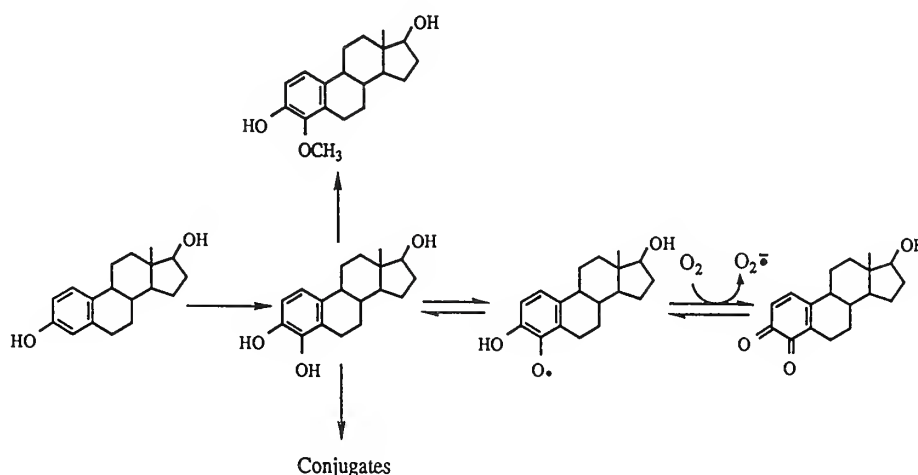


Fig. 8. Metabolic redox cycling between hydroquinone or quinone forms of 4-hydroxyestradiol (as shown) or 2-hydroxyestradiol (not shown). Either catechol estrogen (CE) may be oxidized to its respective quinone by organic hydroperoxide-dependent P4501A enzymes. The quinones, in turn, may be reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytochrome P450 reductase or other reductases. The semiquinone form is an intermediate in both oxidation and reduction reactions and may react with mo-

lecular oxygen to form superoxide radicals and quinone. Superoxide radicals may be reduced to hydrogen peroxide and further to hydroxy radical in the presence of metal ions. Although both CEs have the ability to undergo metabolic redox cycling, hydroxy radical damage has been observed mainly with 4-hydroxylated estrogens, presumably because elevated concentrations of these metabolites exist in target tissues where estrogens induce tumors.

form stable or unstable DNA adducts, as described in Chapter 4. The semiquinone free radical is an intermediate in each of these conversions. The estrogen semiquinone may react with molecular oxygen and form quinone and superoxide radicals (98). Thus, metabolic redox cycling is a mechanism of metabolic activation resulting in the continuous formation of free radical species from possibly small amounts of CE substrate (Fig. 8).

Superoxide radicals may be reduced enzymatically or nonenzymatically to hydrogen peroxide and further to hydroxy radicals, which are capable of initiating lipid peroxidation. Lipid hydroperoxides are, thus, formed by metabolic redox cycling of CEs and, in turn, support the formation of CEs and their redox cycling. This is achieved by lipid hydroperoxides functioning as cofactors for the organic hydroperoxide-dependent estrogen-2-/4-hydroxylase (78,86) and for CYP1A1, which catalyzes the oxidation of hydroquinones, including CEs, to corresponding quinones (99). This shift toward lipid hydroperoxides as cofactors for enzyme-catalyzed reactions represents a loss of metabolic control in the cell, which is exerted in part by the bioavailability of NADPH as cofactor for biosynthetic reactions. Therefore, metabolic redox cycling of CEs, including 4-OHE₂, may persist indefinitely in an unregulated fashion. Moreover, this redox cycling process amplifies damage because relatively small amounts of CEs may repeatedly undergo this process and generate greater than stoichiometric amounts of various radical species.

Conclusions

4-OHE₂ is a carcinogenic metabolite and may be formed by four different processes. 4-Hydroxysteroids may be generated: (a) as a minor byproduct of NADPH-dependent 2-hydroxylation of either E₁ or E₂ due to a lack of specificity of the estrogen-2-hydroxylases; (b) by specific NADPH-dependent 4-hydroxylation of E₁ or E₂ catalyzed by CYP1B1 and possibly other 4-hydroxylases; (c) by organic hydroperoxide-dependent estrogen-2- and 4-hydroxylase activity, which forms these two catechols in approximately equal amounts [little is known about the location and regulation of this enzyme(s)]; and (d) by deconjugation of 4-methoxysteroids and other CE conjugates. Tumors are expected to arise in cells or tissues that experience high concentrations of 4-OHE₂ or 4-OHE₁ formed by one or several of the processes cited above. High concentrations of 4-hydroxysteroids may be found in cells with high formation of CEs by aromatic hydroxylation of parent estrogens, inadequate phase II metabolism of these catechols, or high rates of deconjugation of phase II metabolites back to CEs. Thus, CE metabolite concentrations are the result of a balance of several processes of formation and catabolism and may be unique for a given cell type, depending on its specific profile of metabolizing and catabolizing enzyme activities.

Elevated concentrations of 4-hydroxylated estrogens may be formed as a result of a loss of regulatory control of cells because organic hydroperoxides may be cofactors for the formation and metabolic redox cycling of CEs. In other cell types, 4-OHE₂ may be formed by specific 4-hydroxylation of E₂. The unique distribution of estrogen-4-hydroxylases in tissues, such as the uterus, breast, and others, points to 4-OHE₂ as a hormone with characteristics distinct and different from those of E₂. Specific 4-OHE₂-induced processes, such as blastocyst implantation in the uterus of mice (100), support this view. Therefore, the organ-specific distribution of E₂-4-hydroxylases may be a function of the role of this catechol in physiologic processes.

Tumors may be formed in organs and cells, which experience a loss of regulatory control as metabolism is converted to an organic hydroperoxide-dependent process from an NADPH-dependent process. Tumors may also arise in cells that express estrogen-4-hydroxylase activity and limited phase II metabolism, as may occur in mammary or uterine cells. More research is needed to examine the metabolic characteristics of each cell type in these organs and, thus, their potential for high CE concentrations and for elevated tumor risk.

ESTROGEN 4-HYDROXYLATION CATALYZED BY HUMAN CYTOCHROME P4501B1: OXIDATIVE METABOLISM OF ESTROGENS

The oxidative metabolism of estrogens *in vivo*, especially E₂ and E₁, is known to occur at several positions, including carbons C-1, C-2, C-4, C-6, C-7, C-11, C-14, C-15, C-16, and C-18 [reviewed in (52,67)]. Studies of the routes of formation of these estrogen metabolites comprise an active area of research in several scientific fields, including endocrinology, pharmacology, environmental health sciences, oncology, and epidemiology. Additional knowledge of the rates of formation, metabolic fate (including further metabolism and routes of elimination), and activities of these hydroxylated steroids will support the scientific assessment of the relative importance of such metabolic pathways to hormonal carcinogenesis and other hormone-related diseases.

The mechanism of carcinogenesis of estrogens has been primarily attributed to their specific action as steroid hormone receptor agonists, controlling cellular growth and differentiation in estrogen-responsive tissues through concerted gene regulation. As discussed more fully in other sections of this monograph (see Chapters 3 and 4), increasing evidence of an additional mechanism of carcinogenesis has focused attention on the CE metabolites, which are less potent estrogens than E₂ yet are biologically reactive, capable of directly or indirectly damaging protein, lipid, and DNA (101-103). Worthy of reiteration, our present knowledge of the mechanisms of estrogen-related disease processes indicates a strong estrogen receptor-mediated action. While the activities and potencies of the various estrogen metabolites are less understood, it is reasonable to predict, and in certain cases known, that the activities of these metabolites range from inactive to toxic to protective. Regarding estrogen-related cancers, the routes and rates of formation of certain estrogen metabolites, specifically the 2-, 16 α -, and more recently the 4-hydroxysteroids, are being investigated as etiologic factors. Of particular interest to this research focus group (The Cancer Cube) and a major topic of the conference preceding this monograph are the local production and activity of 4-OHE₂.

4-Hydroxylated metabolites represent only a small percentage of the total amount of estrogens detected in the urine, and 4-hydroxylation was previously considered a minor metabolic route (104). However, tissue 4-hydroxylation of E₂ may play an important role in estrogen homeostasis. In human (87) and mouse (80) uteri, rat pituitary (79), and hamster kidney (105), the rate of E₂-4-hydroxylation equals or exceeds the rate of 2-hydroxylation, and, notably, these organs are sites of estrogen-induced tumors (62,81,106,107). In comparison to normal tissue, elevated E₂-4-hydroxylase activity has been observed in human tissue samples prepared from breast (88,108) and uterine (87) tumors. Furthermore, in male hamster kidney, the carcinogenic and DNA-damaging activity of 4-OHE₂ and the lack of activity

of 2-OHE₂ (83,85,109) implicate the 4-hydroxylated metabolites in estrogen carcinogenesis (11). Evidence of the toxicity of CE via the generation of free radicals through lipid hydroperoxide-supported (110) reductive-oxidative cycling mechanisms continues to accumulate (111–113). Furthermore, several laboratories (71,114,115) have demonstrated that estrogen-3,4-quinones can form DNA adducts, indicating the direct genotoxic potential of these compounds. Requisite to elucidating the contributions of the 4-hydroxysterogens to estrogen carcinogenicity are the identification and characterization of the enzymes that produce these metabolites.

IDENTIFICATION AND CHARACTERIZATION OF HUMAN CYP1B1

A novel human cytochrome P450 was first isolated by differential hybridization as a TCDD-responsive complementary DNA (cDNA) clone from a human keratinocyte cell line treated with TCDD. Levels of this messenger RNA (mRNA) (P4501B1) were shown to be increased 50-fold by treatment with 10 nM TCDD, in part as the result of increased rates of gene transcription (116). Analysis of the complete cDNA sequence of this 5.1-kilobase (kb) TCDD-inducible mRNA identified a new gene subfamily of cytochrome P450, CYP1B1, based on 40% sequence homology to other polycyclic aromatic hydrocarbon (PAH)-inducible isoforms, CYP1A1 and CYP1A2 (90). The CYP1B1 gene was mapped to human chromosome 2 by polymerase chain reaction (PCR) amplification of human/rodent somatic cell hybrid panels using specific primers to the 3'-untranslated region of the CYP1B1 cDNA. Segregation analysis of the data showed 100% concordance between the presence of the PCR product specific for CYP1B1 and chromosome 2 and greater than 8% discordance for all other chromosomes. Southern blot analysis of human genomic DNA, using a single cDNA probe corresponding to the 5'-portion of the CYP1B1 open reading frame, suggested the presence of only a single gene in this subfamily (90).

In the 1980s through studies on the metabolism of PAH, Jefcoate and co-workers (117) found that several rodent tissues, including embryo fibroblasts, adrenals, and the mammary gland, produced anomalous product ratios suggesting the presence of a novel P450 cytochrome. This was established 10 years later by the purification of a novel P450 that was found in each of these tissues. Subsequently, Jefcoate and co-workers tried to address the function of this form, notably by characterizing its expression pattern and regulation. They used antibodies raised against the purified protein to identify expression of equivalent human forms in human breast cells and keratinocytes (118), as well as in a variety of rodent steroidogenic cells (adrenal, testis, ovary) (119). Expression in drug-metabolizing organs, like the liver, was low. This suggested that the function was physiologic in these nonhepatic tissues (i.e., the mammary gland). This was supported by the strong hormonal regulation demonstrated in steroidogenic cells. The induction of this P450 cytochrome by PAH was established, suggesting involvement of the aryl hydrocarbon (Ah) receptor. Cloning of the mouse and rat cDNAs from mouse embryo fibroblasts and rat adrenals, respectively (91,120), completed the initial characterization of this P450. This characterization allowed Jefcoate's laboratory to confirm that this rodent P450 had the same characteristics of and high sequence homology (82%) to human CYP1B1.

In human tissues, Northern blot analysis of CYP1B1 expres-

sion showed that CYP1B1 mRNA could be detected in each of 15 different tissue RNA samples prepared from heart, brain, placenta, lung, liver, skeletal muscle, kidney, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. In primary cultures of normal human epidermal keratinocytes treated for 24 hours with 10 nM TCDD, levels of CYP1B1 mRNA were increased more than 70-fold (90), demonstrating that TCDD induction of CYP1B1 expression in keratinocytes was not restricted to the transformed cell line SCC-12F (116).

Isolation and initial characterization of the human P4501B1 gene (121) described the DNA sequence of a 12-kb genomic clone corresponding to the entire 5.1-kb CYP1B1 cDNA (90) and containing 3.0 kb of upstream (5'- of the ATG) DNA (123). Comparison of these sequences revealed the presence and positions of three exons (371, 1044, and 3707 base pairs [bp]) and two introns (390 and 3032 bp), with the CYP1B1 open reading frame spanning exons 2 and 3. Southern blot analysis using cDNA probes corresponding to each of the three exons of CYP1B1 supported the presence of only a single CYP1B1 gene and excluded the existence of pseudogenes (121). High-resolution chromosome mapping confirmed the previous somatic cell hybrid analysis (90) and further mapped the CYP1B1 gene to human chromosome 2 at 2p21–22 (121). A single transcription initiation site was identified in this CYP1B1 gene, which lacks a consensus TATA box sequence. Deletion analysis of CYP1B1-promoter reporter-gene constructs identified a specific region (–1022 to –835) containing three TCDD-responsive enhancer-core-binding motifs (5'-GCGTG-3') contributing to the TCDD-inducible expression of CYP1B1 in the human keratinocyte cell line SCC-12F (121).

Comparison of the human CYP1B1 genomic and cDNA sequences, obtained independently from two cell lines derived from different individuals, revealed three sequence differences (allelic variants, potential polymorphisms). Concurrent human genetic studies to identify one of two loci for primary congenital glaucoma (PCG) led to the mapping of the PCG locus GCL3A to human chromosome 2 at the 2p21 region [reviewed in (122)]. Subsequent investigations (123–125) have led to independent reports that identify distinct CYP1B1 gene mutations that segregate with the GCL3A phenotype in PCG families. GCL3A-linked PCG has an autosomal recessive mode of transmission, and it appears that the observed mutations will result in the absence of P4501B1 protein and/or activity. Nonetheless, further understanding of this disease will require additional knowledge of the physiologic function of CYP1B1.

As an added benefit of the extensive DNA sequence analysis of the translated regions of the CYP1B1 gene in 22 PCG families and in 100 randomly selected normal individuals (124), the Val 432–Leu CYP1B1 polymorphism (90,121) was confirmed. Three additional polymorphisms predicting variant amino acid sequences were identified: Arg 48–Gly, Ala 119–Ser, and Asn 453–Ser. The frequency of each wild-type allele, calculated for the reported sample of 100 normal individuals (124), is as follows: Arg 48, 0.71; Ala 119, 0.71; Val 432, 0.28; and Asn 453, 0.76. The identification of several frequently occurring polymorphisms in the human CYP1B1 gene demonstrates the strength of direct genomic sequence analysis as a method to identify single nucleotide polymorphisms. At this time, the appropriate procedures to determine the functional significance of such variant proteins are less clear, making this an active area of research.

Nonetheless, epidemiologic studies, similar to those reported for catechol-*O*-methyltransferase polymorphisms and the risk for breast cancer (126,127), are under way for CYP1B1. Such studies should further clarify the role of CYP1B1, specifically, and hydroxylated estrogen metabolites, generally, in estrogen-related diseases.

ESTROGEN HYDROXYLATION CATALYZED BY HUMAN P4501B1

The widespread clinical use of antiestrogens in the adjuvant treatment of breast cancer provided a strong rationale for studies to determine the mechanism(s) of the antiestrogenic activity of ligands for the Ah receptor (128,129). In the MCF-7 breast tumor cell line, a sensitive gas chromatography/mass spectrometry method of analysis was used to show that treatment with TCDD resulted in large, more than 10-fold, increases in the rates of hydroxylation of E_2 at positions C-2, C-4, C-15 α , and C-6 α (129). These studies support the role of increased E_2 metabolism in the observed antiestrogenic effects of TCDD, which include the inhibition of estrogen-mediated expression of tissue plasminogen-activator activity and the formation of multicellular foci (130,131). Spink et al. (130) convincingly demonstrated that P4501A1 catalyzed the hydroxylation of E_2 at the C-2, C-15 α , and C-6 α positions in MCF-7 cells treated with TCDD. However, the metabolism at the C-4 position was shown to be distinct, catalyzed by an unknown TCDD-inducible CYP, best characterized as a low- K_m E_2 -4-hydroxylase.

Isolation and characterization of the TCDD-inducible CYP1B1 from humans (90,116) and from rodent species (91,120,131) and the observation that human CYP1B1 mRNA was elevated by treatment of human breast cell lines with TCDD led to studies of CYP1B1 expression and activity in MCF-7 cells (89). Antibodies raised against mouse CYP1B1 (anti-P450-EF) and cross-reactive with human CYP1B1 (117,118) were used to investigate E_2 hydroxylation catalyzed by microsomes prepared from TCDD-treated MCF-7 cells. This antibody preparation was shown to selectively inhibit E_2 -4-hydroxylation in a concentration-dependent manner. Furthermore, the elevated expression of P4501B1 protein and mRNA in these TCDD-treated cells closely paralleled the observed rates of E_2 4-hydroxylation (89). Collectively, these results provided strong support for the assignment of human CYP1B1 as the low- K_m E_2 -4-hydroxylase that is induced in MCF-7 human breast cancer cells by TCDD treatment.

To establish the specific relationship between the P4501B1 gene product and E_2 metabolism, human P450 protein was expressed in *Saccharomyces cerevisiae* (3). Two recombinant P4501B1 expression plasmids were described. One construct, identified as CYP1B1 Δ 0, was designed to express a protein of 543 amino acids, corresponding to the entire deduced amino acid sequence of P4501B1. A second expression construct, identified as CYP1B1 Δ 3, was designed to produce a protein that did not contain the three amino acid residues after the initial methionine (deletion of amino acids 2–4). The design of the second plasmid was based on the reported amino acid sequence of rat P4501B1, showing that this protein did not contain the first four amino acid residues of its corresponding amino acid sequence (120). Levels of P4501B1 protein present in the microsomal fraction of the CYP1B1 Δ 3 construct (340 pmol/mg protein) were about 10-fold greater than the levels of P450 of CYP1B1 Δ 0 preparations (3). This difference in expression of P450 may be important, since a

similar construct containing this same deletion of amino acids 2–4 also resulted in the highest expression levels of human P4501B1 in *Escherichia coli* (132). Further studies of the translation, processing, and subcellular localization of this protein are warranted. Yeast CYP1B1 Δ 0 microsomal preparations, containing the lower P4501B1-specific content, were determined to have the greatest E_2 hydroxylation turnover numbers; these rates were not increased by the addition of NADPH cytochrome P450 reductase to the reaction mixture. Reactions containing these microsomes were shown to catalyze the 4- and 2-hydroxylation of E_2 , with K_m values of 0.71 and 0.78 μ M and turnover numbers of 1.39 and 0.27 nmol product/minute per nmol P450, respectively (3).

The major CEs detected in serum and urine are the 2-hydroxylated metabolites. The liver is the primary site of estrogen metabolism, where rates of 2-hydroxylation, catalyzed by P4501A2, 3A3, and 3A4, greatly exceed the rate of 4-hydroxylation. The reported apparent K_m values for E_2 hydroxylation catalyzed by the human forms of these enzymes range from 20 to 156 μ M, which are considerably higher than the apparent K_m values of P4501B1, less than 1 μ M (Table 4) (3,132–137). The turnover number for the formation of 4-OHE $_2$ by P4501B1 is similar to the turnover numbers for the formation of 2-OHE $_2$ by human P4501A2, 3A3, and 3A4. The E_2 -4-hydroxylase activity of P4501B1 has the highest catalytic efficiency (turnover/ K_m) of any reported estrogen hydroxylase, and the apparent K_m values of E_2 -4- and 2-hydroxylase activities are the lowest reported values for estrogen hydroxylation (Table 4) (3). Comparisons of the values presented in Table 4 indicate a potential role of P4501B1 hydroxylase activity in estrogen homeostasis, especially in extrahepatic organs, which express much lower levels of P4501A2 and 3A4 than does the liver (138).

In further experiments to explore the significance of the low- K_m 4-hydroxylase activity of P4501B1, cellular E_2 metabolism was studied in MCF-7 cells treated with the potent Ah receptor ligand, indolo[3,2*b*]carbazole (ICZ) (139). MCF-7 cells exhibited ICZ concentration-dependent increases in P4501B1 and 1A1 mRNA levels. In parallel experiments to determine E_2 metabolism, treatment of MCF-7 cells with 10 μ M ICZ for 72 hours, followed by replacement of medium containing 1 μ M E_2 for 6 hours, resulted in a TCDD-like profile of E_2 metabolites (129), with increased rates of hydroxylation occurring at the C-2, C-4, C-6 α , and C-15 α positions of E_2 ; the rates of 2-hydroxylation were the greatest, approximately threefold to sevenfold greater than the rates of hydroxylation at the other positions. When the E_2 concentration was decreased to 10 nM, the rates of 2- and 4-hydroxylation in ICZ-treated cells were detectable and approximately equal, demonstrating that the E_2 4- and 2-hydroxylation activities of P4501B1 are significant at low, physiologically relevant concentrations of E_2 (3). In summary, these studies demonstrate that human P4501B1 is a catalytically efficient E_2 -4-hydroxylase that is likely to participate in endocrine regulation and estrogen-related disease.

Tissue Expression of Human P4501B1

Large differences are demonstrated between species in CYP1B1 metabolism of PAH (5,140). Thus, the question arose whether the expression of CYP1B1 in breast cells gives any indication of physiologic or pathologic functions linked to such activity. The relative expression levels of CYP1A1 and CYP1B1 will determine the conversion of E_2 to the 2- and 4-hydroxyl-

Table 4. Activities, kinetic parameters, and catalytic efficiencies of human estrogen hydroxylases*

	2-Hydroxylation			4-Hydroxylation			16 α -Hydroxylation			Reference No.
	K_m , μM	Turnover, k (min ⁻¹)	ce, k/ K_m (sec ⁻¹ · M^{-1})	K_m , μM	Turnover, k (min ⁻¹)	ce, k/ K_m (sec ⁻¹ · M^{-1})	K_m , μM	Turnover, k (min ⁻¹)	ce, k/ K_m (sec ⁻¹ · M^{-1})	
<i>Estradiol hydroxylation</i>										
CYP1B1	0.78	0.27†	2.1×10^7	0.71	1.39†	1.2×10^8		ND		(3)
CYP1A2	20	11	3.3×10^7	28	0.9	1.9×10^6	58	0.7	7.2×10^5	(135)
	32	3.3‡	6.2×10^6		NR			NR		(136)
CYP3A4	54	0.8	8.9×10^5	111	0.3	1.6×10^5	75	0.4	3.2×10^5	(135)
	156	3.3‡	1.3×10^6		NR			NR		(136)
CYP19	1.58	0.45	1.7×10^7		NR			NR		(137)
<i>Estrone hydroxylation</i>										
CYP1B1		NR			NR			NR		
CYP1A2	19	9.2	2.9×10^7	17	2.0	7.1×10^6		<0.1		(135)
	14	5.43	2.3×10^7		<0.1			<0.1		(138)
CYP3A4	102	0.7	4.1×10^5	78	0.6	4.6×10^5	64	0.5	4.7×10^5	(135)
		<0.2			<0.2		95	0.68	4.3×10^5	(138)

*Activities and kinetic parameters were determined as described in the referenced manuscripts. Catalytic efficiencies (turnover/ K_m) were calculated with the use of these reported values. ND = not detected; NR = not reported; K_m = Michaelis constant; ce = catalytic efficiency; k = turnover.

\ddagger Shimada et al. (132) reported similar turnover numbers for recombinant P4501B1 purified from *Escherichia coli* (2-hydroxylation, 0.13 min^{-1} ; 4-hydroxylation, 1.4 min^{-1}).

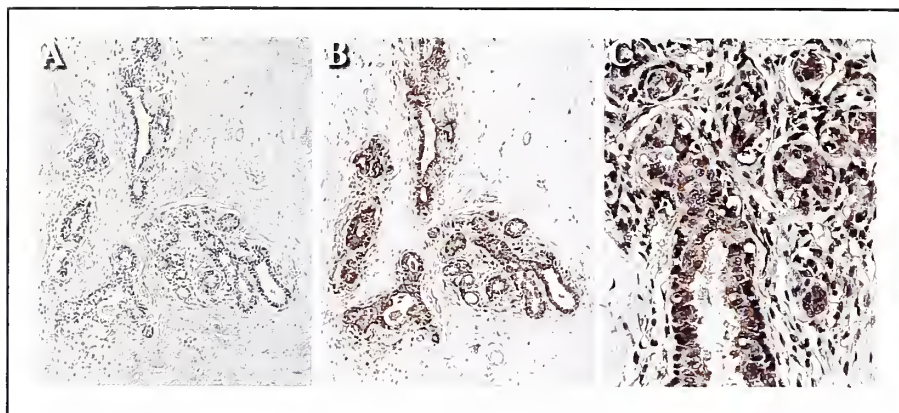
\ddagger Aoyama et al. (137) reported similar turnover numbers for CYP1A2 (2-hydroxylation, 2.74 min^{-1} ; 4-hydroxylation, 0.27 min^{-1}) and for CYP3A4 (2-hydroxylation, 1.30 min^{-1} ; 4-hydroxylation, 0.31 min^{-1}).

ation products, respectively. CYP1B1 is expressed constitutively in cultured breast luminal epithelial cells, which are the source of most breast tumors (5). This implies that there will be a low basal formation of 4-CE in all tumors. Typically, CYP1A1 is essentially undetectable in breast tissue from most donors. Environmental activators of the Ah receptor, such as polychlorobiphenyls, which have been detected at considerable levels in breast fat, could potentially induce both forms. This certainly is the case for these chemicals in cultured breast epithelial cells (5). However, analysis of CYP1A1 mRNA in human breast tissue by reverse transcription-PCR (RT-PCR) indicated that this isoform is typically undetectable. Low levels are occasionally present in cultured epithelial cells, but this is retained independent of medium changes and is almost certainly constitutive. These data indicate that relatively little 2-CE will be produced *in vivo*. A central question to be addressed and resolved in future research is whether this very low formation of 4-CE is physiologically or pathologically significant. The relatively high affinity of CYP1B1 for E_2 allows this activity to be retained even at low physiologic hormone levels. While it might seem that this activity is too low to remove E_2 , a substantial proportion of CYP1B1 is localized in nuclear and perinuclear membranes. This may exert a relatively greater effect on nuclear levels of E_2 and 4-CE production. This constriction to depletion of estrogen will depend on the relative activity of other metabolic processes acting on E_2 . CYP1B1 seems to be the major source of 4-CE formation, and the measurement of this steroid *in vivo* points to substantial accumulated activity.

Information on the "basal" and inducible expression of P4501B1 in human cells and tissues continues to be an interesting, yet less developed, aspect of the knowledge of this recently identified CYP. Additional knowledge of tissue P4501B1-specific content and activity will strengthen scientific assessments of the relative importance of estrogen metabolic pathways to estrogen-related diseases and their prevention.

As stated above, the initial characterization of human P4501B1 revealed that levels of this 5.1-kb transcript were detectable in multiple adult tissue samples, the kidney sample exhibiting the greatest apparent signal relative to the other samples tested (90). In a subsequent study (4), these results were extended to include analyses of additional adult tissue samples representing five additional organs and a second kidney sample, comparative analyses of the relative expression of P4501A1 and 1A2 in these same tissue samples, and analyses of tissue samples from five fetal organs. P4501A1 mRNA was detected in 12 of the 21 adult tissue RNA samples but in none of the five fetal tissue RNA samples. The most intense hybridization signals occurred in the prostate and mammary tissues. P4501A2 mRNA was detected only in the adult liver sample. P4501B1 mRNA was detected in 20 of the 21 adult RNA samples (heart, brain, placenta, lung, liver, skeletal muscle, kidney 1, kidney 2, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, adrenal, pituitary, uterus, and mammary tissue) and in fetal heart, brain, lung, and kidney. The levels of P4501B1 RNA in adult kidney of both sexes and fetal kidney, as well as in prostate, uterus, and mammary tissue, were apparently greater than those in other tissues (4). Many of these findings were confirmed in a report describing an RT-PCR-based analysis of P4501B1 expression showing that human P4501B1 is expressed mainly in extrahepatic tissues of adults and fetuses (141). Contrary to the earlier report (4), P4501B1 RNA was not detected in samples of adult lung (141). While the basis of this discrepancy is unlikely to be identified, other investigators (142) have reported the detectable expression of this mRNA in lung tissue samples from smokers. In summary, these preliminary analyses of the organ-specific RNA distribution of the P4501 gene family have revealed that, while P4501A2 is expressed primarily in the liver, P4501A1 and 1B1 are expressed widely and found in many of the same tissues. Furthermore, P4501B1 appears to be the pre-

Fig. 9. Immunohistochemical analysis of P4501B1 protein in breast tissue of a nonpregnant woman of reproductive age. Photomicrographs are at a magnification of $\times 100$ (A and B) or $\times 400$ (C). **Panel A:** preimmune immunoglobulin G (IgG). **Panels B and C:** anti-P4501B1 IgG. The analysis was performed as previously described (146), except that the concentration of the primary antibody was 0.3 $\mu\text{g/mL}$. The secondary antibody was a goat anti-rabbit streptavidin-conjugated antibody, and the staining was detected using biotin-conjugated horseradish peroxidase (HRP) complex and subsequent development using diaminobenzidine as the chromogen. The slides were lightly counterstained with hematoxylin.



dominant family 1 P450 expressed in human fetal tissues (4,141).

Regarding the expression of P4501B1 in organs such as breast and uterus, where strong associations between estrogen exposure and the risk for cancer are known, accumulating evidence indicates the presence and activity of this enzyme in these tissues. Elevated CE production has been associated with tumors of the breast (88,108,143,145), and P4501B1 RNA (4,145,146) and protein (147) have been detected in both normal (4,146) and tumor (145–147) breast tissue samples. Furthermore, in human uterine tissue, where 4-hydroxylation of E_2 is increased in myomas compared with surrounding myometrium, the 4-hydroxylase activity of myoma microsomes was strongly inhibited by an antimouse P4501B1 antibody, suggesting that, in human uterine tissue, E_2 4-hydroxylation is catalyzed by P4501B1 (87). In addition, Larsen et al. (5) demonstrated the expression of P4501B1 protein and activity in early-passage human mammary epithelial cells isolated from reduction mammoplasty tissue of seven individual donors. Specific contents of CYP1B1 and CYP1A1 protein in microsomal preparations (day 6 of culture) were quantitated by immunoblot analysis. Levels of constitutive CYP1B1 protein ranged from 0.01 to 1.4 pmol/mg microsomal protein; exposure to TCDD increased these levels, ranging from 2.3 to 16.6 pmol/mg microsomal protein. Levels of constitutive CYP1A1 protein were much lower than those of CYP1B1. However, the inductive response of CYP1A1 to TCDD treatment was very strong, resulting in comparable specific contents of the two P450 cytochromes in microsomes prepared from treated cells. This study indicates that human mammary epithelia constitutively expresses variable levels of functional CYP1B1 protein (11), which may contribute to the oxidative metabolism of E_2 and other estrogens.

To aid in the analysis of CYP1B1 protein expression, our laboratory has produced polyclonal rabbit anti-P4501B1 antibodies that were shown to be both sensitive and CYP1B1 specific, detecting this protein by both immunoblot and immunohistochemical analyses (147,148). Recently, we (149) have used these antibodies to investigate the constitutive expression and cellular localization of P4501B1 in normal human tissue samples. A representative immunohistochemical analysis of P4501B1 protein expression in normal human breast tissue is presented in Fig. 9 (Sutter TR, Kim J, Sherman M: manuscript in preparation). As best seen in Fig. 9, panels B and C, P4501B1 is expressed ubiquitously in the ducts of this breast lobule, conspicuously present in the epithelia, as well as the myoepithelia, stromal fibroblast, and endothelial cells. P4501B1 staining is

predominantly cytoplasmic, yet some nuclear staining is evident. Like the previous study (5), these results are consistent with the concept that human breast epithelial cells constitutively express significant amounts of CYP1B1 protein, which may contribute to the oxidative metabolism of E_2 and other estrogens in this tissue.

Conclusions

The kinetic parameters determined for the E_2 -4-hydroxylase activity of human P4501B1 establish this enzyme as the most catalytically efficient estrogen-hydroxylase described to date. This observation is important because it suggests that this enzyme is responsible for the E_2 -4-hydroxylase activity that has been observed in several tissues, such as human uterus and breast. The specific expression of P4501B1 and formation of CEs have been independently associated with estrogen-related tumors in multiple tissues and species. Further elucidation of the physiologic and/or pathologic significance of the E_2 -4- and 2-hydroxylase activities of P4501B1 will require additional knowledge of the tissue, cellular, and developmental expression (and regulation thereof) of this gene, and its protein product in humans and other animals. Despite the present knowledge gaps, our understanding of human P4501B1 as a low- K_m E_2 -4- and 2-hydroxylase and a major extrahepatic form of CYP firmly supports the hypothesis of the role of CE metabolites in estrogen-related carcinogenesis.

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NOTES

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Chapter 6: Estrogen Metabolism by Conjugation

Rebecca Raftogianis, Cyrus Creveling, Richard Weinshilboum, Judith Weisz

The involvement of estrogens in carcinogenic processes within estrogen-responsive tissues has been recognized for a number of years. Classically, mitogenicity associated with estrogen receptor-mediated cellular events was believed to be the mechanism by which estrogens contributed to carcinogenesis. Recently, the possibility that estrogens might contribute directly to mutagenesis resulting from DNA damage has been investigated. That damage is apparently a result of the formation of catechol estrogens that can be further oxidized to semiquinones and quinones. Those molecules represent reactive oxygen species and electrophilic molecules that can form depurinating DNA adducts, thus having the potential to result in permanent nucleotide mutation. Conjugation of parent estrogens to sulfate and glucuronide moieties; of catechol estrogens to methyl, sulfate, and glucuronide conjugates; and of catechol estrogen quinones to glutathione conjugates all represent potential “detoxification” reactions that may protect the cell from estrogen-mediated mitogenicity and mutagenesis. In this chapter, the biochemistry and molecular genetics of those conjugative reaction pathways are discussed. When applicable, the involvement of specific enzymatic isoforms is presented. Finally, the activity of many of these conjugative biotransformation reactions is subject to large interindividual variation—often due to the presence of common nucleotide polymorphisms within the genes encoding those enzymes. Functionally significant genetic polymorphisms that might contribute to variable conjugation of estrogens and catechol estrogens are also discussed. [J Natl Cancer Inst Monogr 2000;27:113–24]

The involvement of estrogens in carcinogenic processes within the breast has been appreciated for a number of years (1–3). The classical concept of estrogens as carcinogens recognizes the mitogenicity of estrogens via estrogen receptor (ER)-mediated cellular events (1). More recently, as has been detailed throughout this monograph (Chapters 3–5), the role of catechol estrogens (CEs) as genotoxic chemical procarcinogens, independent of ER mediation, has been recognized (2–4). Although estrogens and CEs differ with regard to the role of the ER in mediating their carcinogenicity, they have in common the potential for “detoxification” via enzyme-mediated conjugation to glucuronide, glutathione (GSH), methyl, and/or sulfate moieties (2). In this chapter, we will discuss primary estrogen and CE conjugation reactions, with particular emphasis on the biochemistry and molecular genetics of the human enzymes that catalyze those reactions.

Estrogens exert biologic responses in steroid hormone-responsive cells largely via interaction with ERs, members of a superfamily of nuclear hormone receptors that act as ligand-activated transcription factors (5). There are two known ER subtypes, ER α and ER β , which share similar estrogen affinities but have dissimilar expression patterns and response to antiestrogens (5–7). The two most potent endogenous estrogens, es-

trone and 17 β -estradiol, are both ligands for the ERs, although those receptors have higher affinity for 17 β -estradiol than for estrone and it is 17 β -estradiol that is believed to be the predominant endogenous activator of ER-mediated cellular processes (5). The most abundant circulating estrogen, however, is the sulfate conjugate of estrone (8,9). The process by which estrogens, synthesized and secreted predominantly by the ovaries, are transported to and exert their biologic effects in steroid hormone target tissues is not completely understood. As will be discussed in this chapter, estrogen conjugates, particularly estrone sulfates, are believed to play an important role in that process (9–11).

Chemical carcinogenesis emerged as a scientific discipline approximately 50 years ago (12,13). One of the principles of that discipline is that compounds often require metabolic “activation” to form genotoxic and carcinogenic metabolites (12,13). That process involves the establishment of a balance between “activating” and “inactivating” metabolic pathways. The hypothesis that estrogens might contribute to the pathophysiology of breast cancer as direct genotoxins (3,4) has raised the possibility of just such a balance between estrogen activation and inactivation in those hypothetical genotoxic effects. Specifically, oxidative reactions, often catalyzed by isoforms of the cytochromes P450, can result in the formation of CEs from parent estrogens and, subsequently, semiquinones and quinones derived from CEs that are capable of forming either stable or depurinating DNA adducts (14–16). Countering the effects of these pathways of metabolic activation are enzymatic reactions that inactivate the parent estrogens, the CEs, and quinones. Inactivation pathways involving conjugation reactions, such as methylation, sulfation, glucuronidation, or conjugation with GSH, will be detailed in this chapter. It is important to note that, although a number of animal models have been developed to facilitate the study of CE-mediated carcinogenesis, the focus of this chapter will be primarily on the conjugation of estrogens and CEs in humans. Although it has been hypothesized that conjugated CEs may exhibit biologic activity (2), the focus of this chapter is on conjugation as a detoxification mechanism.

Finally, conjugation pathways of both estrogens and CEs display large variations among individuals—often as a result of common genetic polymorphisms. Therefore, the possibility arises that common, inherited variations in enzymatic pathways for estrogen bioactivation or in the inactivation of either the parent compound or downstream metabolites might represent individual risk factors for the occurrence of breast cancer. The

Affiliations of authors: R. Raftogianis, Department of Pharmacology, Fox Chase Cancer Center, Philadelphia, PA; C. Creveling, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD; R. Weinshilboum, Department of Pharmacology, Mayo Medical School/Mayo Clinic/Mayo Foundation, Rochester, MN; J. Weisz, Department of Obstetrics and Gynecology, Milton S. Hershey Medical Center, Pennsylvania State University.

Correspondence to: Rebecca Raftogianis, Ph.D., Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111 (e-mail: RB_Raftogianis@FCCC.EDU).

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molecular epidemiology of estrogen carcinogenesis is detailed in Chapter 7. That chapter focuses on genetic polymorphisms that have been studied as risk factors for estrogen-mediated carcinogenesis. This chapter will present the current state of knowledge with regard to functionally significant genetic polymorphisms in human genes encoding estrogen-conjugating enzymes, many of them as yet untested as breast cancer risk factors.

ESTROGEN CONJUGATION

Biologic Role of Estrogen Conjugation

The endogenous formation of estrogen conjugates has long been recognized as a major route of estrogen metabolism (17). Both endogenous and synthetic exogenous estrogens are extensively biotransformed to estrogen conjugates in humans (Fig. 1) (2,18). The most abundant circulating estrogen conjugates are the sulfates, followed by the glucuronides. It is important to note that conjugated estrogens are not appreciable ligands for the ERs; thus, they do not promote ER-mediated activity (2). Intuitively, it was initially assumed that sulfate and glucuronide conjugation of estrogens represented a pathway resulting in less active, more polar, and more readily excreted estrogenic compounds. It is now appreciated, however, that estrogen sulfates actually exhibit a much longer half-life than do the parent estrogens (2,8,11). Estrone sulfate is the most abundant circulating estrogen, at concentrations approximately 10-fold higher than unconjugated estrone (8). That finding, as well as increasing knowledge about the transport and subsequent desulfation of estrogen sulfates, has led to a widely held belief that sulfated steroid hormones serve an important biologic role as steroid hormone precursors, particularly for steroid hormone-responsive tissues (2,9). An increasing body of scientific data supports the hypothesis that sulfation and desulfation of estrogens may well represent an endogenous system important in the regulation of biologically active steroid hormones in target tissues (10,11). Specifically, it is currently hypothesized that inactive estrone sulfate is transported to target tissues via the circulatory system, taken into target cells, most likely by organic anion transporters, enzymatically hydrolyzed to estrone by intracellular membrane-bound steroid sulfatase (arylsulfatase C), and hydroxylated to active 17 β -estradiol via catalysis by 17 β -hydroxysteroid dehydrogenases (2,11,18,19). 17 β -Estradiol activates the ER via ligand binding and initiates a number of downstream ER-mediated events—most notably related to transcriptional activation of those genes that contain DNA sequences that bind and respond to activated ERs (5,18).

The transport of estrone sulfate into steroid hormone-responsive cells is not well understood; however, some studies (19,20) have shown that a human organic anion transporter (Oatp1) has high affinity for both sulfate and glucuronide estrogen conjugates. Furthermore, this transporter is typically responsible for intracellular import of organic ions rather than the efflux of these compounds out of the cell. The level of expression or activity of this transporter in human breast tissues has not yet been reported. Many target tissues including the breast exhibit estrogen sulfation activities in addition to the ability to desulfate estrogen sulfates (8,9). This "cycling" has been demonstrated in mammalian cells and, like phosphorylation and dephosphorylation of proteins during cell-signaling processes, sulfation and desulfation of steroid hormones possibly represent an intracellular regulatory mechanism for estrogenic activity

(Fig. 1) (8,11,21). Recognition of the importance of steroid sulfatase activity in the formation of intratumoral estrogens has resulted in the development of a number of steroid sulfatase inhibitors for the treatment of steroid hormone-responsive tumors (22,23).

Enzymes responsible for the glucuronidation and deglucuronidation of estrogens are also expressed in a variety of human tissues, including the breast (24,25). Estrogen glucuronides have received much less attention, however, than have the sulfate conjugates as steroid hormone precursors, most likely because they are less abundant and more readily cleared from the body (2). Breast tumors and breast cancer cell lines express high levels of β -glucuronidase, the enzyme that catalyzes the hydrolysis of estrogen glucuronides; however, appreciable estrogen glucuronide cycling in breast tissue has not been demonstrated (24). Although the concept of estrogen glucuronides as steroid precursors has been underinvestigated, it is generally accepted that glucuronidation of estrogens serves primarily a classical excretory role. Estrogen glucuronide conjugates are readily excreted in both urine and bile (26).

Biochemistry of Estrogen Conjugation

Sulfation. Sulfate conjugation of estrogens is catalyzed by several members of a superfamily of cytosolic sulfotransferase (SULT) enzymes (27,28). SULT enzymes catalyze the transfer of SO_3^- from 3'-phosphoadenosine-5'-phosphosulfate, the enzymatic cofactor, to, in the case of estrogens, phenolic acceptor groups (28). Cytosolic SULTs are active as homodimers. Sulfation of estrone and 17 β -estradiol occurs at the 3-phenolic

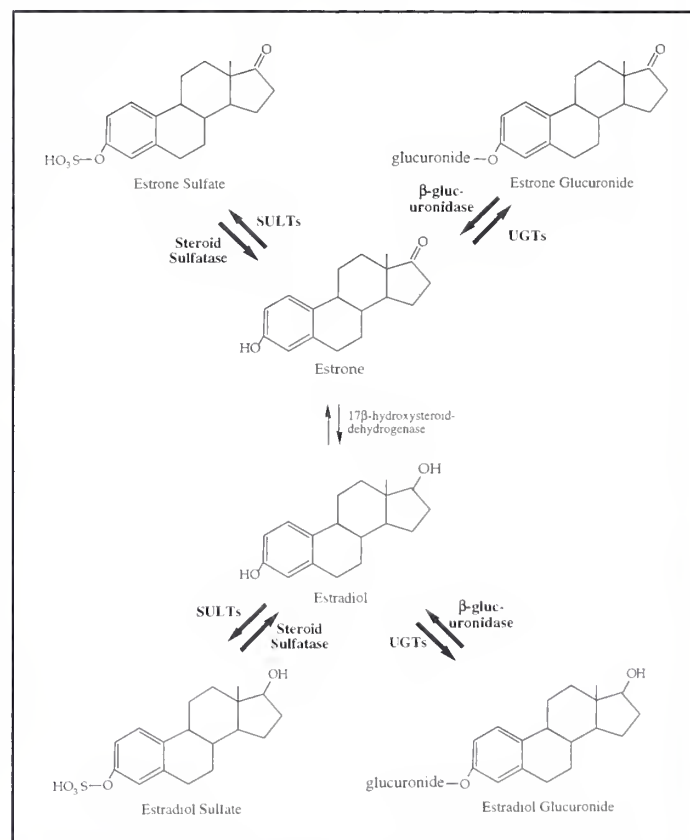


Fig. 1. Conjugative pathways for estrone and 17 β -estradiol. Sulfation, desulfation, glucuronidation, and deglucuronidation reactions catalyzed by sulfotransferases (SULTs), steroid sulfatase, UDP-glucuronosyltransferase (UGTs), and β -glucuronidase, respectively, are shown.

group of the steroidal A ring (Fig. 1). Estrogen SULT activity has been demonstrated in a variety of human tissues, including liver, small intestine, kidney, placenta, uterus, adrenal gland, and breast (29–33). The level of estrogen SULT activity in the human liver is high, and this activity is believed to contribute significantly to the high circulating levels of estrone-3-sulfate (29,30). Although from a quantitative perspective, sulfation of estrogens in the liver is probably the most important overall estrogen-conjugating activity in the body, sulfation of estrogens in steroid target tissues, including the breast, has also been demonstrated and may well be important in affecting the biologic activity of estrogens within those tissues (2,11).

Study of the association of estrogen SULT activity with breast cancer has been an active area of research. Expression of estrogen SULT activity within breast tumors has been reported to correlate with the ER status of the tumor as well as with the response of tumors to estrogens and adrenalectomy (33,34). However, other studies have shown no such correlation or even an inverse correlation (35). Such contradictory findings are indicative of the difficulty investigators have encountered in the study of estrogen sulfation in steroid target tissues. As will be discussed shortly, the reason for those difficulties has recently been appreciated in that we now know that multiple SULT enzymes contribute to estrogen SULT activity and, importantly, there is significant interindividual variation in the level of activity of the enzymes catalyzing the sulfation of estrogens (27,30,36). Furthermore, SULTs are subject to profound substrate inhibition (32). The concentration of substrate at which inhibition occurs differs among estrogen-sulfating isoforms such that slight differences in experimental conditions would have important implications in the interpretation of resulting data.

Glucuronidation. Estrogen glucuronidation is catalyzed by several members of a superfamily of microsomal UDP-glucuronosyltransferase (UGT) enzymes (25,37). UGTs catalyze the conjugation of UDP-glucuronic acid, the UGT cosubstrate, to a variety of endogenous and exogenous aglycones, including steroid hormones (38). Whereas estrogens are sulfated predominantly at the 3 position, glucuronidation can occur at either the 3 or 17 β hydroxyl group of steroidal hormones, with the 17 β position being the apparent predominant site of glucuronidation for 17 β -estradiol (Fig. 1). Glucuronidation of estrogens renders those molecules less lipophilic and more readily excreted in both urine and bile. 17 β -Glucuronides of estradiol are known to induce cholestasis, putatively via interaction with hepatocyte canalicular membrane efflux transporters such as MDR1 and MRP2/cMOAT (39,40).

Steroid hormone glucuronidation has been observed in human liver, biliary epithelium, kidney, gut, prostate, ovary, and breast (25,26,38). In a study comparing UGT activity in matched breast ductal carcinoma and peritumoral tissues, the authors (24) reported activity in tissues from only four of the 12 individuals studied. Furthermore, in those four sample pairs, the level of activity was fivefold lower in tumor tissue than in the peritumoral tissue. However, those studies were conducted with the use of 4-methylumbelliferone as substrate (as opposed to an estrogen), and it is not clear whether that activity correlates with estrogen glucuronidation in the breast. Glucuronidation is a major route of androgen metabolism, and the study of this pathway has received much attention in terms of its role in the pathophysiology of androgen-dependent diseases (41). However, the role of estrogen glucuronides in breast cancer has received little

attention compared with sulfate conjugation. This is most likely due to the perception that estrogen glucuronidation serves a predominantly excretory role, secondary to sulfate conjugation. It is clear that much further study of the glucuronidation of estrogens is required before we can fully understand the biochemistry of this pathway and its role in affecting estrogen activity.

Molecular and Cellular Aspects of Estrogen Conjugation

Biochemical studies of estrogen conjugation provided much knowledge about these important metabolic pathways. However, there were also many questions left unanswered by these studies, and we now have begun to be able to answer some of those questions using the tools and further knowledge gained with the advent of molecular biology. There are a surprising number of SULT and UGT isoforms capable of contributing to the conjugation of estrogens. Those isoforms are often expressed in a tissue-specific manner and are often under specific regulatory control. Furthermore, a number of those conjugative enzymes are encoded by genes known to harbor common genetic polymorphisms. These factors help explain many of the complexities of estrogen conjugation—and this knowledge allows us to probe estrogen conjugation reactions in a systematic fashion.

Sulfotransferases. The cloning of SULT genes and complementary DNAs (cDNAs) is a very active area of research (27). Currently, there are at least 10 unique cytosolic SULT enzymes known to be expressed in human tissue (27,42–45). On the basis of amino acid sequence identity, those 10 human SULTs fall within two families, SULT1 and SULT2. Subfamilies include SULTs 1A, 1B, 1C, 1E, 2A, and 2B. The 1A, 1C, and 2B families each have multiple members. Although amino acid identity allows the classification of these enzymes into families and subfamilies, members exhibit overlapping substrate affinities even across families. Estrone and 17 β -estradiol are substrates for SULT1A1, SULT1E1, and SULT2A1, although the affinity of these enzymes for estrogens varies (Table 1) (27). Overlapping substrate specificity of SULTs toward estrogens complicates the study of estrogen sulfation. For example, the high affinity of SULT1E1 for estrogen substrates suggests that this enzyme plays a major role in the endogenous sulfation of estrogens, and the activity of this enzyme in the liver likely contributes significantly to the quantitatively large pool of circulating estrogen sulfates (29). It would be logical to hypothesize that this enzyme activity might be important in regulating estrogen activity in breast tumors. However, studies have suggested that, although SULT1E1 appears to be expressed in normal breast epithelial cells, it is not highly expressed in breast tumors or cell lines derived from breast tumors (46). SULT1A1 and, to a lesser extent, SULT2A1 appear to be the SULT isoforms primarily responsible for estrogen sulfation in breast tumors (46–48). These findings suggest that a specific SULT isoform may play a variable role in endogenous steroid hormone sulfation, depending on the tissue and the disease of interest.

Relatively little is known about the regulation of SULT genes. Although genes have been cloned for most of the human SULT cDNAs and enzymes identified to date, the DNA sequences contributing to the promotion or regulation of transcription of those genes have not been well defined. Of the human SULT genes cloned thus far, only SULT1E1 contains a canonical TATA box element, and experimentally determined sites of transcription initiation appear to correspond to the location of

Table 1. Specificity of sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) isoforms with various estrogen and catechol estrogen substrates

Isoform	Substrate*					
	Estrone	Estradiol	2-OH-estrone	4-OH-estrone	2-OH-estradiol	4-OH-estradiol
SULTs						
1A1	X	X	ND	X	X	X
1E1	X	X	ND	ND	ND	ND
2A1	X	X	ND	—	—	X
UGTs						
1A1	—	X	X	X	X	X
1A3	X	ND	X	X	X	X
1A4	—	X	—	—	X	X
1A7	—	—	ND	—	X	ND
1A8	X	ND	X	X	X	X
1A9	X	X	X	X	X	X
1A10	X	X	ND	X	X	ND
2B4	—	—	ND	X	X	ND
2B7	—	—	X	X	X	X

*X = isoform has been shown to conjugate indicated substrate; — = isoform has been shown not to conjugate the indicated substrate; ND = interaction of the indicated isoform/substrate pair has not been determined. See text for details and references.

that element (49). The level of estrogen SULT activity in human tissues has been reported to be under the influence of steroid hormones (28). In concordance with that finding, the 5'-flanking region of the SULT1E1 gene contained half palindromic glucocorticoid and thyroid hormone response elements (49). However, the functional significance of those elements has not yet been studied experimentally. Additional evidence of SULT regulation includes the identification of alternative sites of transcription initiation for the SULT1A1 gene (50). The regulation or tissue selectivity of alternative transcriptional initiation of SULT1A1 has not been well studied.

Finally, conjugation of estrogens is known to vary significantly among individuals (29,51). That observation raises the possibility that genetic variation in the genes contributing to estrogen conjugation (pharmacogenetics) may contribute to interindividual variation in estrogen metabolism. As will be discussed in Chapter 7, a number of genetic variants in genes contributing to estrogen metabolism have been reported to represent risk factors for the development of breast cancer. The study of the pharmacogenetics of SULT enzymes is currently an active endeavor. A common, functionally significant genetic polymorphism has been described for SULT1A1 (52,53). That SULT1A1 polymorphism results in an Arg213His amino acid substitution. Correlation of the level of SULT activity in human blood platelet samples and SULT1A1 genotype suggests that individuals homozygous for the His allozyme exhibit a significantly diminished capacity to sulfate prototypic phenolic molecules (52). The contribution of this polymorphism to interindividual variability in the conjugation of estrogens or as a risk modifier for breast cancer has not yet been reported.

Similarly, large interindividual variations in the level of SULT2A1 activity in human liver and the level of immunoreactive protein in intestinal tissues have also been reported (30,54). Genetic polymorphisms resulting in Met57Thr and Glu186Val amino acid changes in SULT2A1 have been reported (55). Functional studies of the recombinant SULT2A1 allozymes (55) have shown that those amino acid changes, particularly when coexpressed, result in a diminished level of recombinant enzyme activity. However, SULT2A1 genotype does not appear to correlate significantly with the level of apparent SULT2A1 activity in human tissues (55). Finally, the presence

of large differences in the level of immunoreactive SULT1E1 protein in samples of human small intestines raises the possibility that genetic polymorphisms might also exist for that enzyme (30). That possibility is currently the subject of active study, but no polymorphisms in the SULT1E1 gene have yet been reported.

Glucuronosyltransferases. As with the SULTs, a number of UGT isoforms are now known to contribute to the conjugation of estrogens (Table 1). The degree of contribution of individual UGTs to that activity is not yet well understood, and it is likely that specific isoforms will contribute differently, depending on the tissue and the disease of interest. There are currently at least 12 functional UGT isoforms known to be expressed in human tissues (37). Like the SULTs, those 12 enzymes fall into two families within the human UGT superfamily of microsomal enzymes. Glucuronidation of estrone and 17 β -estradiol appears to be catalyzed by several members of the UGT1 family. Thus far, recombinant human UGTs 1A1, 1A3, 1A4, 1A8, 1A9, and 1A10 have all been shown to catalyze the glucuronidation of estrone and/or 17 β -estradiol (Table 1) (25,26,56–60). It is interesting to note that, for some human recombinant UGT isoforms, there appears to be selective affinity for estrone or 17 β -estradiol as substrate. For example, UGTs 1A1 and 1A4 exhibit activity toward 17 β -estradiol but not toward estrone, whereas UGTs 1A9 and 1A10 have been reported to catalyze the glucuronidation of both of these estrogens (25,26,56,58,60). Activity for UGTs 1A8 and 1A3 toward estrone has been reported, but the activity of those isoforms toward 17 β -estradiol has apparently not been evaluated (57,59).

There is much known about the expression of human UGT1A isoforms in various tissues. It should be noted, however, that the tissue distribution profile of some UGT isoforms has not been as extensively characterized as others. UGT1A1 is expressed in human liver, colon, and biliary epithelium (gallbladder) but not in stomach (60). UGT1A3 has been reported to be expressed in human colon, biliary epithelium, and liver, but the level of expression in liver varied significantly between individuals and was fivefold to 10-fold less than the level of UGTs 1A1 and 1A4 (57,60). UGT1A4 is expressed in human liver, colon, and biliary epithelium but not in stomach (60). UGT1A8 appears to be expressed specifically in human intestinal tissues (59,60). UGT1A9 is expressed in human prostate, testis, breast, ovary,

skin, skeletal muscle, stomach, small intestine, colon, liver, and kidney but not in biliary epithelium or stomach (25,60). UGT1A10 is expressed in colon, biliary epithelium, and stomach but not in liver (60). It is important to note that, although only the UGT1A9 isoform has been reported to be expressed in human breast to date, that is likely a reflection of the lack of evaluation of the level of expression of various UGT isoforms in that tissue.

The regulation of the UGT1 family is currently not well characterized but is an active area of study. The most notable feature of this gene family is that all of the UGT1A isoforms disseminate from a single "nested" gene structure (37). There are six coding exons in the human UGT1A genes, and each isoform is encoded by the same exons 2 through 5. The only differentiation between isoforms is that each exon 1, encoding the N-terminal half of the protein, is unique, and isoform specificity results from alternative transcription initiation and usage of unique exons 1 (37). Therefore, each isoform is under the control of individual promoter sequences, and isoform-specific regulation has been observed. For example, as noted in the previous paragraph, UGT1A isoforms are differentially expressed in human tissues.

As previously noted, the capacity for estrogen conjugation and, specifically, glucuronidation is known to vary widely in the human population (51). That observation raises the possibility that genetic variation may exist in the UGT isoforms that contribute significantly to estrogen glucuronidation. A functionally significant common polymorphism in the promoter sequence of the UGT1A1 gene has been described and well characterized (61–63). That polymorphism is a variable length (TA)_n TAA repeat in the functional TATA box upstream of exon 1 of the UGT1A1 gene. The wild-type allele is defined as n = 6. Allelic variants identified to date include n = 5, 7, and 8 (63). *In vitro* studies utilizing reporter constructs driven by allelic variants of the UGT1A1 promoter (63) have shown that promoter activity appears to decrease with increasing n. Furthermore, clinical association of the most common variant (n = 7) with a relatively poor ability to glucuronidate bilirubin (Gilbert's syndrome), as well as the chemotherapeutic agent SN-38, has been observed (61,64). Studies determining the association of UGT1A1 alleles with estrogen metabolism and risk modification of breast cancer have not yet been reported, but they will be of great interest.

CE CONJUGATION

Biologic Role of CE Conjugation

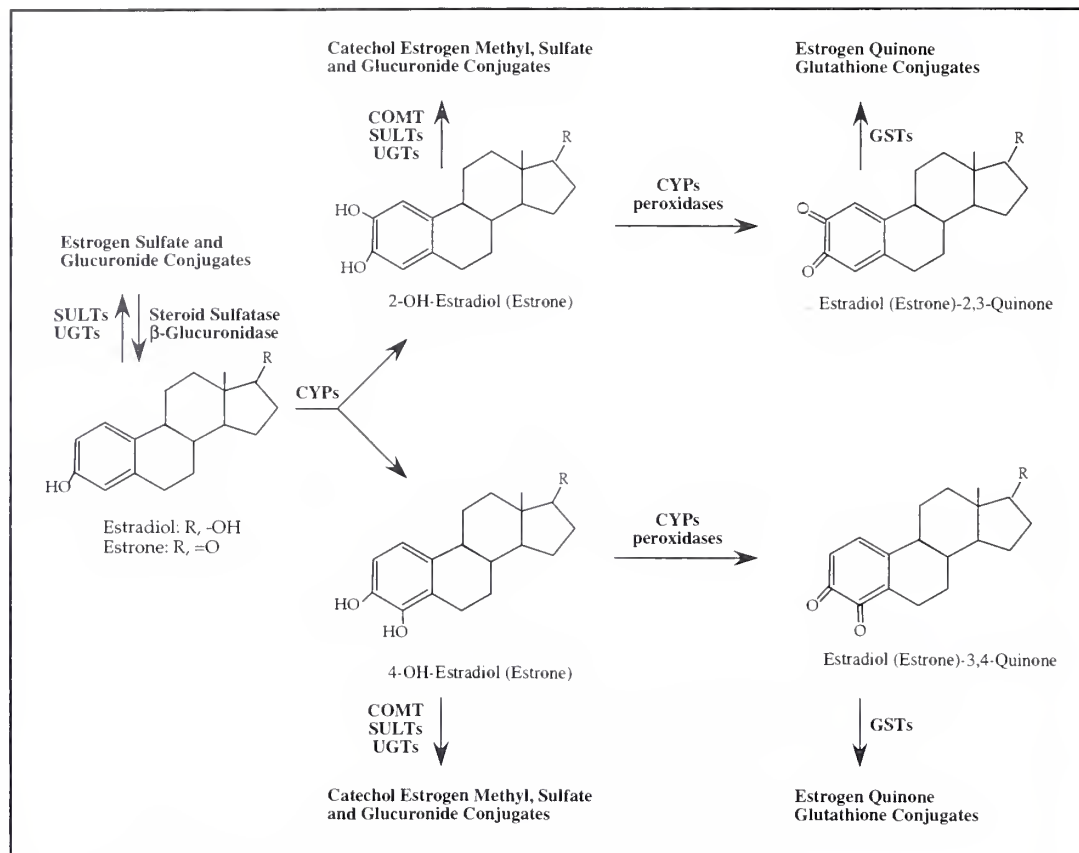
The putative role of CEs in the mediation of breast carcinogenesis has been described in Chapters 3–5 of this monograph. The biotransformation of estrone and estradiol to CEs involves hydroxylation at the 2 or 4 position of the steroidal A ring of these parent estrogens (3,14). Those reactions are catalyzed by multiple cytochrome P450 isoforms. Both the 2- and the 4-hydroxy CEs can be further oxidized to CE quinones (CE-Qs) or semiquinones (Fig. 2) (16). The 2-hydroxy CE-Qs have been shown to form stable DNA adducts, whereas the 4-hydroxy CE-Qs have been shown to form depurinating adducts (16,65,66). There is good evidence suggesting that those depurinating adducts can lead to apurinic DNA sites and permanent mutations that, when inflicted upon critical DNA sequences, can lead to tumorigenesis (16,66). CEs can also enter into redox cycling

and, thereby, become a source of reactive oxygen species (3). Hence, unless CEs are inactivated, they may contribute to carcinogenesis by causing DNA damage mediated by reactive oxygen species and by direct interaction of CE-Qs with DNA to form depurinating adducts (65,66). Fortunately, our cells are fortified with an armament of conjugative pathways that result in the biotransformation of toxic estrogen metabolites to relatively nontoxic moieties (2). Generally, the reactive CEs are detoxified by biotransformation to predominantly methyl conjugates, to a lesser extent glucuronides, and possibly sulfate conjugates (Fig. 2) (2). A further conjugative safeguard lies in the detoxification of CE-Qs via conjugation to glutathione (16,67). Therefore, the actual risk of CEs in causing DNA damage may well depend on the ability of individual cells to conjugate CEs and CE-Qs relative to the rate of formation of these toxic estrogen metabolites. In the rest of this chapter, we will focus on CE conjugative pathways and those conjugative enzymes responsible for the detoxification of CEs and CE-Qs.

The most well-studied CE conjugation reaction is that of methylation. CE methylation is catalyzed by catechol-O-methyltransferase (COMT), an enzyme that exists in both "soluble" (S-COMT) and membrane-bound (M-COMT) forms, as discussed in detail below (16,68). Studies in the hamster kidney (69) provided the first example linking an estrogen-induced cancer with the induction of COMT. The localization of COMT in the epithelial cells of the proximal convoluted tubules of the hamster kidney is similar to its localization in the rat kidney reported earlier (70). Hamsters treated with primary estrogens, such as estrone and estradiol, develop tumors in the renal cortex. There is evidence that the carcinogenicity of estrogens for hamster kidney results from a combination of factors: 1) an increase in the catechol load, 2) the presence of high levels of 2- and 4-hydroxylated CEs subject to oxidative metabolism in the renal cortex, and 3) a relative insufficiency of COMT (71). In control hamsters, COMT was localized in the cytoplasm of proximal convoluted tubules, predominantly in the juxtamedullary region where estrogen-induced tumors arise. After 2 or 4 weeks of treatment with estrogen, COMT was seen in epithelial cells of the proximal convoluted tubules throughout the cortex. Moreover, many cells showed intense nuclear COMT immunoreactivity (Fig. 3) (69). The estrogen-induced cancers were COMT negative but were surrounded by tubules with epithelial cells with intense cytoplasmic and nuclear immunostaining. Immunoblot analysis indicated that the nuclear COMT, shown in Fig. 3, was S-COMT. This translocation to the nucleus was shown by sequencing of hamster kidney COMT messenger RNA to occur in the absence of a nuclear localization signal. This pattern of induction of COMT in hamster kidney in response to estrogen treatment, in particular in the nucleus, has been interpreted as a possible response to "a threat" to the genome by products of oxidative metabolism of CEs.

It is of interest that nuclear localization of COMT is not unique to hamster kidney but also can be seen in some normal, as well as neoplastic, mammary epithelial cells (72). Human breast tissues have the capacity to synthesize both 2- and 4-hydroxyestrogens (71,73). A cytochrome P450 that catalyzes the 2- and 4-hydroxylation of estrogen has been identified by immunocytochemistry in human ductal epithelial cells (74). COMT has also been identified in those cells (75). High levels of oxidatively damaged DNA have been found in breast tissue from women in the United States (76,77). It is reasonable to propose

Fig. 2. Conjugation of estrogens, catechol estrogens, and estrogen quinones. Reaction pathways and the enzymes involved are shown. For simplicity, the formation of estrogen semiquinones is not depicted. (See Chapter 5 for details.) COMT = catechol-*O*-methyltransferase; SULTs = sulfotransferases; UGTs = UDP-glucuronosyltransferases; CYPs = cytochromes P450; GSTs = glutathione *S*-transferases.



that, in human breast tissue, like the kidneys of hamsters treated with estrogen, oxidative metabolism of CE might contribute to this oxidative damage.

Biochemistry of CE Conjugation

Methylation. Quantitatively, the most active CE conjugative pathway is methylation. CE methylation is catalyzed by COMT, a member of a superfamily of methyltransferase enzymes (68). COMT, a classical phase II enzyme, catalyzes the transfer of methyl groups from *S*-adenosyl methionine, the enzyme cofactor, to hydroxyl groups of a number of catechol substrates, including the CEs. Under normal circumstances, CEs are, for the most part, promptly *O*-methylated by COMT to form 2- and 4-*O*-methylethers, which are then excreted (78). While virtually all catechols are substrates for COMT, the highest affinities for the enzyme are exhibited by the CEs (78). The existence of this metabolic pathway helps to explain the extremely short half-life of CEs and the predominance of *O*-methylethers of CEs, in particular of 2-methylethers, as the major metabolites of estrone and estradiol in urine (79). However, under circumstances during which the capacity for *O*-methylation is reduced or inhibited by an excess catechol load, the half-life of CEs may be extended. This phenomenon could have special importance for specific cellular sites, such as breast epithelial cells, where CEs are formed. COMT might play an important role in protecting the genome from damage that could be caused by the metabolism of estrogens through activation of the CE-Q pathway. A number of investigators are now studying the involvement of this enzyme as well as the interindividual variability of COMT enzyme activity in detoxification of CEs specifically in the context of breast carcinogenesis.

The hypothesis that COMT provides a protective mechanism against cytotoxicity and genotoxicity by preventing the oxidation of catechols is in its infancy. At present, we know enough to consider *O*-methylation an important mechanism for preventing cytotoxic and genotoxic damage caused by products of the oxidative metabolism of catechols. This knowledge may generate avenues for therapeutic intervention where a deficit in the capacity for *O*-methylation appears to be a risk factor in carcinogenesis (80,81).

Sulfation and glucuronidation. While methylation of CEs has been well studied, very little is known about the role of sulfation and glucuronidation in the detoxification of CEs. The excretion of both sulfate and glucuronide conjugates of CEs has been observed in rats (82), and it is clear from a number of *in vitro* studies that UGTs and SULTs are able to catalyze the conjugation of CEs. We also know that those enzymes are expressed in the liver and estrogen-responsive tissues, such as the breast epithelium (25,27). Therefore, it is plausible to suggest that these reactions may play a biologic role in the detoxification of CEs. A number of studies [reviewed in (2)] have reported that the major urinary metabolites of CEs are the methyl conjugates. From a quantitative perspective, therefore, the formation of sulfate and glucuronide CEs does not appear to represent major pathways in the overall metabolism and excretion of CEs. However, because the reactivity and toxicity of the CEs are intracellular phenomena, it has been suggested that local metabolism of CEs within target cells will be just as important as the overall detoxification of CEs in the liver (2). For that reason, a number of investigators are now studying the role of sulfate and glucuronide conjugation of CEs in the intracellular detoxification of these carcinogens. COMT, UGTs, and SULTs often share affin-

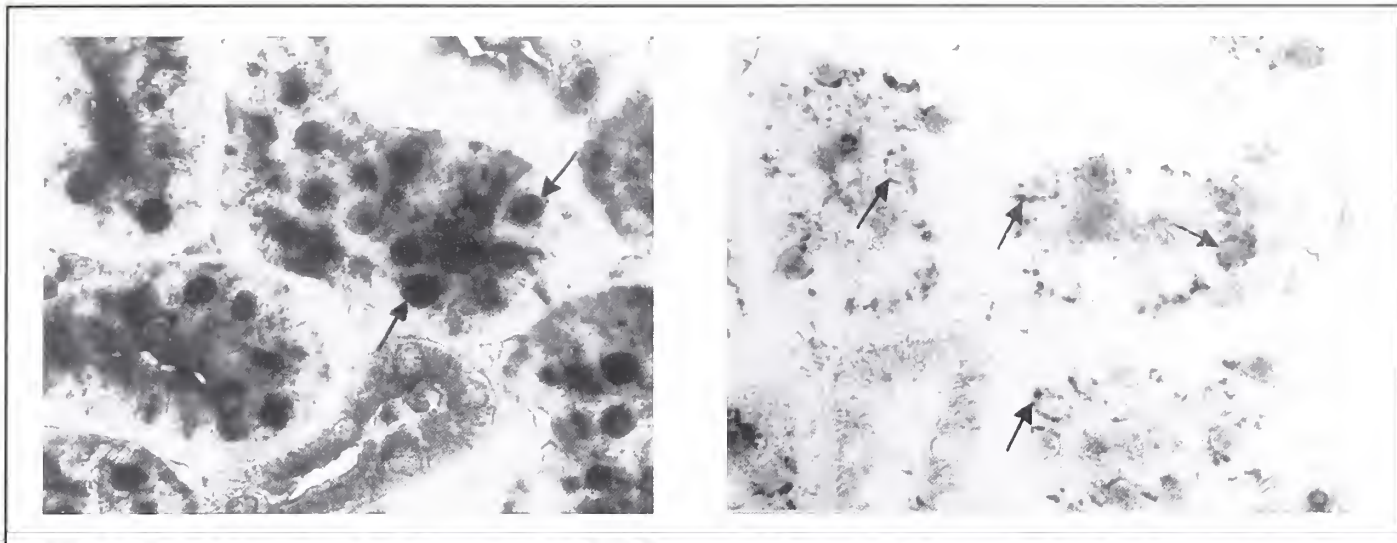


Fig. 3. Immunoreactive catechol-*O*-methyltransferase (COMT) (**left**) and CuZn-superoxide dismutase (CuZnSOD) (**right**) in proximal convoluted tubules from the same region in adjacent tissue sections from the kidney of a hamster treated for 2 weeks with estradiol (original magnification $\times 500$). Intense immunostaining for COMT is seen with many nuclei in contrast to the perinuclear immunostaining for CuZnSOD. **Arrows** point to some of the cells with distinct immunostaining for COMT and perinuclear staining for CuZnSOD. This figure is used by permission of Oxford University Press from *Carcinogenesis* (69).

ity for the same substrates. An indirect role that has been suggested for the relevance of SULTs and UGTs in the detoxification of CE is that those enzymes might represent pathways for the conjugation of other catechol substrates that may compete for, and thus inhibit, the capacity of COMT to detoxify CE (2). Those hypotheses have yet to be rigorously tested at the experimental level.

GSH conjugation. The reactivity of CE-Qs relates to their ability to undergo redox cycling, creating oxidative stress, and/or to react directly with cellular nucleophiles (such as DNA) (3,16). Conjugation of quinones to GSH, a major cellular sulfhydryl tripeptide, is generally considered a detoxification mechanism (16,83). GSH conjugation of CE-Qs has been shown to occur both *in vivo* and *in vitro* (84). GSH-conjugated CE-Qs are then rapidly converted to mercapturic acid metabolites that are readily excreted from the cell. It is primarily this excretory role of GSH conjugation that is believed to contribute to the detoxification of CE-Qs. However, the actual degree of detoxification of CE-Qs that is imparted by GSH conjugation is unclear because GSH-conjugated quinones are capable of undergoing the same redox cycling reactions as are the parent quinones and semiquinones (83,84). Those reactions result in the formation of reactive oxygen species that can themselves cause DNA damage. Therefore, the "net" protective effect of conjugation of CE-Qs by GSH depends on the relative balance between GSH-mediated CE excretion and the GSH-mediated formation of reactive oxygen species. Most studies appear to confirm that conjugation of CE-Qs with GSH results in a net decrease in DNA damage (67,85).

Molecular and Cellular Aspects of CE Conjugation

Catechol-*O*-methyltransferase. A single gene encoding COMT is expressed at the protein level in two forms as a consequence of the existence of alternative transcription initiation sites (86). The two transcriptional products result in the translation of an S-COMT and an M-COMT enzyme with M_r values of 23 000 and 26 000 daltons, respectively. M-COMT includes

an additional 50 amino acid residues at the N-terminus of the protein that are not present in S-COMT (86,87). Of the two forms, the cytosolic S-COMT has a lower affinity but a higher capacity for catecholamines than M-COMT (68). The relative expression of the two COMT enzymes varies with different tissues, but S-COMT appears to be the dominant form in most cell populations (87–89). COMT is widely distributed, with high levels of activity being reported in the liver and kidney epithelium, as well as in the ependymal and glial cells. In breast tissue, immunoreactive COMT has been observed in both normal and neoplastic epithelial cells (75). In neoplastic cells of rodent and human breast, COMT enzyme activity, expressed as units per milligram of protein, has been reported to be elevated (75,90). However, this apparent increase may be due to an increase in cell numbers in neoplastic breast parenchyme.

Extensive cytochemical studies of the localization of COMT both at the cellular and subcellular levels (91) support the hypothesis that COMT plays a critical role in the local regulation of catechols at specific target sites. Regulation of COMT expression appears to be tissue selective and site specific. In liver and possibly in red blood cells, COMT functions in the *O*-methylation of circulating endogenous and xenobiotic catechols (92). In addition, in liver, quantitatively the most important site for the metabolism of estrogens via 2-hydroxylation, COMT serves to inactivate 2-OH CE close to the site where they are formed. In many other tissues in which COMT is expressed, it appears to have a critical role in restricting the passage of catechols between tissue compartments (93). An example is the dense concentration of COMT in the epithelial cells of the choroid plexus that separate the vascular system from the spinal fluid. Another example is the presence of COMT in ependymal cells lining brain ventricles separating the spinal fluid from the brain parenchyma. The presence of COMT in astrocytes, oligodendrocytes, and microglia may well restrict the movement of catechols to "fields" in the central nervous system. In certain tissues, the expression of COMT has been shown to be under hormonal control.

Studies of the expression of COMT in the rat uterus provide

an example of a precise spatial and temporal expression of COMT and of its hormonal regulation in relation to a critical physiologic event, implantation (94). Immunoreactive COMT becomes evident in the luminal epithelium of the uterus at the site of decidualization just before implantation on day 3 of pregnancy. The role of progesterone in the induction of COMT was demonstrated by the effective blockade of enzyme expression by RU-486 (95). Since there is evidence that CEs generated in the uterus may have an important role in the process of implantation, the induction of COMT by progesterone could serve to delimit the action of CEs to the implantation site (96).

Finally, levels of COMT activity in humans were shown more than 20 years ago to be controlled, in part, by a common genetic polymorphism (97). The phenotypic trait of low COMT activity was found in approximately 25% of a Caucasian population. Molecular pharmacogenetic studies (98) have identified a single nucleotide polymorphism in the COMT gene that results in a Val108Met (amino acid 108 in S-COMT) amino acid substitution. This amino acid change is of great functional significance, since the methionine substitution results in a protein with low enzyme activity, and correlation of low COMT activity with COMT genotype has been reported in human tissues. It is notable that this COMT genetic variant represents a truly "balanced" polymorphism, in that the frequency of occurrence of each allele is approximately 50%. The description of the molecular genetic basis for low COMT activity made possible genetic epidemiologic studies and, as pointed out in Chapter 7, COMT has been a focus for studies of the genetic epidemiology of breast cancer. Unfortunately, the results of those studies (80,81,99) are conflicting. Therefore, these two complementary issues serve to illustrate—in both a broad and a highly focused fashion—the promise and limitations of this overall research strategy. This approach almost certainly will be applied with increasing frequency to help elucidate the possible contribution of direct estrogen genotoxicity to the pathophysiology of breast cancer and other neoplasia.

Sulfotransferases and UDP-glucuronosyltransferases. The role of sulfation and glucuronidation as detoxification pathways for CEs is underinvestigated. However, catalysis of CE conjugation by human recombinant SULT and UGT enzymes has been reported. Those results will be presented here, but it should be cautioned that the relevance these studies have to *in vivo* CE conjugation is not yet clear. SULT1A1, in addition to catalyzing the sulfation of estrone and estradiol, also catalyzes the sulfation of 4-hydroxyestrone, as well as of 2- and 4-hydroxyestradiol (Table 1) (100). In that same study, SULT2A1 was reported not to catalyze the sulfation of 2-hydroxyestradiol or 4-hydroxyestrone but to have marginal activity toward 4-hydroxyestradiol. There have apparently been no other reports of specific SULTs catalyzing the sulfation of CEs.

It is tempting to speculate that SULT1A3, a catechol-preferring SULT, or SULT1E1, an estrogen SULT, might participate in the sulfation of CEs. Reverse transcription-polymerase chain reaction studies have suggested that SULT1A3 was highly expressed in human breast tumors and cell lines relative to the expression of SULT1A1 (Raftogianis R; unpublished data). However, it is not yet known whether SULT1A3 contributes to the sulfation of CEs. It has also been suggested that SULT1A3 might indirectly contribute to the regulation of CE conjugation by sulfating other catechols that would otherwise compete for COMT (2), thus inhibiting CE methyl-

ation. Hypotheses involving the role of SULT1A3 in CE conjugation have yet to be rigorously tested experimentally. SULT1E1 is expressed in normal breast epithelium, but it is not known whether that enzyme catalyzes the sulfation of CEs (46).

A common polymorphism has been described for SULT1A1 (52,53), and a number of laboratories are currently testing the hypothesis that this polymorphism may represent a risk factor for breast cancer. SULT1A1 polymorphisms are hypothesized to modify susceptibility to estrogen-mediated carcinogenesis via both sulfation of parent estrogens and variable detoxification of CEs (Figs. 1 and 2). Finally, biochemical pharmacogenetic studies (101) have shown that a common genetic polymorphism results in interindividual variation in the activity of SULT1A3. However, there have been no reports on the molecular basis for this polymorphism. Should SULT1A3 be involved in the detoxification of CEs, it is possible that polymorphisms in this gene might represent risk factors for susceptibility to CE-mediated breast cancer.

A large number of human recombinant UGTs, from both the UGT1 and UGT2 families, catalyze the glucuronidation of CEs (Table 1). Although there is much substrate overlap among these isoforms, there does appear to be some selectivity of isoforms toward specific CEs. UGT1A1 and UGT1A3 both catalyzed the conjugation of 2- and 4-hydroxy CEs, with particularly high activity toward the 2-hydroxy CEs (102). UGT1A4 exhibited low levels of activity toward 2- and 4-hydroxyestradiol and no activity with estrone CEs (58). UGT1A7 has been shown to catalyze the glucuronidation of 2-hydroxyestradiol (60). UGT1A8 and UGT1A9 have also been reported to conjugate all four CEs, but with particularly high activity toward the 4-hydroxy CEs (25,59). In a separate publication (60), however, UGT1A8 was reported not to catalyze the conjugation of 2-hydroxyestradiol or 4-hydroxyestrone. UGT1A10 catalyzed the conjugation of 2-hydroxyestradiol and 4-hydroxyestrone (60). In the UGT2 family, the recombinant enzymes for both UGT2B4 and UGT2B7 catalyzed the glucuronidation of CEs (102–104). UGT2B4 (previously referred to as 2B11) catalyzed the conjugation of 4-hydroxyestrone and 2-hydroxyestradiol (104). UGT2B7 exhibited activity toward the 2- and 4-hydroxy CEs, with particularly high activity toward the 4-hydroxy CEs (102,103).

In addition to the functional variable repeat polymorphism in the TATA box already discussed for UGT1A1, common polymorphisms exist in both UGT2B4 and UGT2B7. The UGT2B4 polymorphism is defined by an Asp458Glu amino acid substitution that results in a protein with diminished UGT activity (105). The UGT2B7 polymorphism causes a His268Tyr amino acid change that apparently does not alter the function of UGT2B7 (102). Whether the UGT1A1 or 2B4 polymorphisms result in clinically significant variation in the *in vivo* conjugation of CEs is not yet known.

Glutathione S-transferases. Members of a superfamily of cytosolic GSTs catalyze the conjugation of GSH, the reactive cosubstrate, to a variety of electrophiles (106). Although GSH conjugation can occur independent of GST-mediated catalysis, GSTs likely play a role in the catalysis of GSH conjugation of CE-Qs. GSTs are a major class of detoxification enzymes. There are estimated to be at least 20 human GST isoforms (106). Their activity has been associated with the inactivation of a large number of xenobiotics, including many drugs. The ability of many tumors to exhibit increased levels of intracellular GST

expression has been implicated as a mechanism of chemotherapeutic drug resistance (107). GST enzymes are encoded by a superfamily of GST genes (106). The nomenclature adopted for this superfamily is quite different from that for the cytochromes P450, SULTs, or UGTs. The five families of GSTs have been designated GST alpha (α), mu (μ), pi (π), sigma (σ), and theta (θ). Humans possess a single functional GST π , but each of the other families contains multiple family members. GST enzymes are active as either homodimers or heterodimers. The frequent occurrence of functional GST heterodimers has made the study of substrate specificity for particular GST isoforms difficult. Perhaps it is for this reason that there is a lack of reports regarding the specific GST isoforms that contribute to the formation of CE-Q-GSH conjugates.

There is much known about the molecular genetics of human GSTs (106). Many GST α isoforms are expressed in human liver and skin, while some are ubiquitously expressed. Some members of the GST μ family are expressed in human liver, while others are expressed in muscle, testis, brain, and heart. GST π is ubiquitously expressed, and GST θ has been reported in human liver and red blood cells. There have been a number of reports indicating the high inducibility of GSTs by a variety of agents. Polycyclic aromatic hydrocarbons, phenolic antioxidants, reactive oxygen species, barbiturates, and synthetic glucocorticoids have been shown to induce GSTs. Induction of GST π has been of particular interest because of its putative role in drug resistance (107). The mechanisms by which GSTs are inducible are apparently diverse (106). The regulation of GST expression appears to be quite complex. A number of genetic response elements have been characterized in GST genes, including xenobiotic, antioxidant, and glucocorticoid-responsive elements. Furthermore, GST subunit expression is quite tissue specific, and regulatory elements contributing to tissue specificity are beginning to be defined. An NF- κ B-like repressor element has recently been described in the human GST π gene. Expression of GSTs also appears to undergo sex- and age-specific regulations.

A number of genetic polymorphisms have been described for human GSTs, including variations in the GST μ , GST π , and GST θ genes (106,108). The most notorious GST polymorphism is the null gene for GST μ (106). This polymorphism is defined by a deletion of the GSTM1 (μ) gene. The frequency of homozygosity of this deletion varies with ethnicity, from approximately 22% in Nigerians to 58% in Chinese populations. Epidemiologic studies have suggested that individuals who are null for the GSTM1 gene may be at increased risk for a variety of neoplastic diseases. The epidemiology of this polymorphism in breast cancer is discussed in Chapter 7 of this monograph. A common single nucleotide polymorphism in the human GST π gene resulting in an Ile105Val amino acid substitution has been identified, and the Val104 variant is associated with low GST π activity (108). In addition, the Val104 allele has been associated with increased risk for prostate cancer. Epidemiologic studies of the role of this polymorphism in breast cancer are discussed in Chapter 7. An additional null allele for a GST θ gene, GSTT1, has also been reported (106). The frequency of the homozygous GSTT1 null genotype has been reported to vary from 16% in British Caucasians to 38% in Nigerians. The biologic consequences of the GSTT1 null genotype are not yet clear, but studies of this polymorphism and breast cancer susceptibility are also discussed in Chapter 7.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Conjugation is clearly a major biotransformation pathway for estrogens in humans. Recognition of the contribution of estrogen conjugation and deconjugation in breast cancer has been a relatively recent event. Increasing evidence suggests that the role of estrogen conjugation, particularly sulfation, goes beyond that of an excretory function and is perhaps even a major regulator of biologically active estrogens. Much less is known about conjugation of CEs, but the role that these conjugative pathways play in the biotransformation of CEs is an emerging story. Methylation of CEs appears to be an important detoxification mechanism, and some evidence suggests that variation in the capacity of cells to methylate CEs may represent a risk factor for susceptibility to breast cancer.

Clearly, more investigative effort will be required to fully understand which, if any, of these conjugative pathways modify cancer susceptibility or progression. As described in the next chapter, the study of low-penetrance, risk-modifying genes is very active, and we are beginning to see the inclusion of genes, such as COMT, that contribute to estrogen and CE conjugation among those being studied. As more genetic polymorphisms in estrogen- and CE-conjugating enzymes are identified, even larger epidemiologic studies will be necessary to delineate which of these variations or—more likely—which set of these genetic variants, represent cancer risk factors. The identification of novel genes encoding conjugating enzymes and “functionally significant” polymorphisms within those genes is occurring at a rapid pace. New molecular information arising from this era of “functional genomics” will require careful biochemical and large-scale epidemiologic studies before we can understand the biologic interplay and ultimate cellular consequence of the apparent myriad biotransformations that estrogens undergo and how these reactions contribute to carcinogenesis.

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NOTES

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Chapter 7: Molecular Epidemiology of Genetic Polymorphisms in Estrogen Metabolizing Enzymes in Human Breast Cancer

Patricia A. Thompson, Christine Ambrosone

Epidemiologic studies indicate that most risk factors for breast cancer are related to reproductive and hormonal factors. For a number of years, the mechanism for estrogens in carcinogenesis was thought to be that of mitotic stimulation, with the growth promotion of ductal epithelial cells harboring precursor mutations in the breast. However, evidence is now available that estrogens may act as initiators of cellular alterations and tumorigenesis. Investigation and measurement of serum levels of estrogens in epidemiologic studies may, therefore, be misleading, because they may reflect levels quite different from those of hormone metabolites to which the target tissue is exposed. Proportions of hormone metabolites may be estimated by evaluation of associations between breast cancer risk and genetic polymorphisms in enzymes involved in hormone metabolism. A number of molecular epidemiologic studies have been conducted to evaluate associations between polymorphic genes involved in steroid hormone metabolism (i.e., CYP17, COMT, CYP1A1, CYP19, GST, and MnSOD) that may account for a proportion of enzymatic variability, and results are discussed in this review. There are strengths and limitations to such an approach, foremost of which may be the lack of insight into the extent to which individual variability in estrogen exposure may be explained by allelic variation. Variability in other endogenous and exogenous factors that impact parent hormones and their metabolites along activation and conjugation pathways may also affect associations in case-control comparisons. This and other possible reasons for inconsistencies in results of molecular epidemiologic studies are discussed. Contributions from population-based studies and those from the laboratory may together move this field ahead and more clearly elucidate the basis of hormonally related cancers, identifying etiologic factors and susceptible populations for preventive strategies. [*J Natl Cancer Inst Monogr* 2000;27:125-34]

Breast cancer is the most commonly occurring cancer among women in the United States, representing 29% of all newly diagnosed cancers in women and is second only to lung cancer as cause of cancer death in women (1). Currently estimated to affect 175 000 women in 1999 (1), a number of putative risk factors for breast cancer have been identified. Of those factors examined in epidemiologic studies, aside from a family history of breast cancer, the majority of risk factors are related to reproductive history and are widely thought to reflect longer lifetime exposures to the endogenous steroid hormones (2). Currently, the hypotheses proposed to explain the role of reproductive risk factors in breast cancer etiology are controversial and are based on the dual effects of estrogens as both promoters of ductal epithelial cell growth (Chapter 8) and as precursors for mutagenic estrogen metabolites [(3-5); Chapters 4 and 5].

Because epidemiologic and animal studies indicate that breast cancer risk may be related to endogenous exposures to steroid hormones, intensive epidemiologic research has been targeted at serum and urinary measurement of parent hormones and their metabolites in both case-control and cohort studies, yielding inconsistent results (3,6). Multiple factors complicate measurement of urinary and serum hormones, including intraindividual variation of hormones as a result of menstrual and diurnal timing, disease status (case-control studies), laboratory variability, and hormone degradation in transport and storage. Furthermore, Zhu and Conney (7) suggest that the measurement of serum estrogens may reflect levels associated with hepatic-specific metabolism that may differ from the local milieu of estrogen metabolites. This supposition about tissue specific differences, as well as the development of approaches to assess tissue-specific exposures in relation to risk require further investigation and are beyond the scope of this discussion (for a discussion of tissue specific metabolism, see Chapter 5).

According to the paradigms that have been developed for studies of bladder and lung cancers with respect to individual susceptibility to chemical carcinogens, metabolic variability that affects xenobiotic metabolism has been suggested by us, and others, to increase the risk of breast cancer among defined subsets of women [reviewed recently in (8,9)]. As observed in drug and chemical metabolism, there is considerable interindividual genetic variability in the metabolic and biosynthetic pathways in steroidogenesis. These person-to-person differences, which are, in part, attributed to allelic variability or gene polymorphisms, might define subpopulations of women with higher lifetime exposures to hormone-dependent growth promotion or to cellular damage from particular estrogens and estrogen metabolites. Such variation could explain a portion of the cancer susceptibility associated with reproductive events and hormone exposure. Currently, the evaluation of associations between breast cancer risk and genetic polymorphisms in enzymes involved in hormone metabolism may be the most effective manner in which to evaluate metabolic variability, until technical and epidemiologic methodologies have been developed to accurately quantify specific estrogens and their metabolites. In this chapter, we will discuss the use of genetic markers in population studies to determine the impact of metabolic diversity in estrogen hormone biosynthesis and metabolism on

Affiliations of authors: P. A. Thompson, Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, Houston; C. Ambrosone, Division of Molecular Epidemiology, National Center for Toxicological Research, Jefferson, AK.

Correspondence to: Patricia Thompson, Ph.D., Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

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breast cancer risk. More important, we will discuss the strengths and limitations of this approach in considering the multigenic nature and complexity of the problem. The powerful introduction of molecular epidemiology as an approach to directly test the role of common allelic variants as risk factors for disease may allow us to determine which steroid hormone pathways are critical determinants of excessive hormonal exposures, and perhaps cancer risk, and ultimately identify targets for preventive strategies.

ESTROGEN BIOSYNTHESIS

Six isoforms of cytochrome-dependent monooxygenase (CYP) are involved in the biosynthesis of various steroid hormones, starting from cholesterol—CYP11A, CYP17, CYP19, CYP11B1, CYP21B, and CYP11B2 (10). As presented in Fig. 1, several enzymes are important in the biosynthesis of estrogens starting from cholesterol. The rate-limiting step in all steroid hormone biosynthesis is the cleavage of the side chain of cholesterol by CYP11A to form the C21 steroids, pregnenolone and progesterone. Hydroxylation and subsequent cleavage of the two-carbon side chain of the C21 steroids by the CYP17 (17- α hydroxylase activity/C17–20 lyase activity) yields the C19 steroids, androstenedione and dehydroepiandrosterone. Androstenedione is the immediate precursor for the formation of testosterone (11). Estrogens are ultimately formed by aromatization of androstenedione and testosterone, catalyzed by the CYP19 (aromatase) (12). In addition to the cytochrome P450, a series of hydroxysteroid dehydrogenases (3 β -HSD and 17 β -HSD) participate in the biosynthesis of the steroid hormones (7,13,14). As depicted in Fig. 1, the 3 β -HSD converts pregnenolone to progesterone, whereas the 17 β -HSD converts androstenedione to testosterone. There are several forms of 17 β -HSD isoforms, and

current data indicate that each form has a distinct role in the metabolism of steroid hormones. 17 β -HSD type 1 and CYP19 catalyze the end steps in 17 β -estradiol (E_2) biosynthesis through androstenedione, supporting a role for this particular isoform in the biosynthesis of E_2 .

ESTROGEN METABOLISM

Once formed, estrogens are extensively metabolized by a number of oxidative and conjugative reactions that can lead to their deactivation and subsequent elimination [(7,15,16); Chapters 5 and 6]. Alternatively, oxidation and conjugation reactions of estrogens may generate metabolites that have distinct biologic activities, including altered hormonal properties; genotoxicity, through the formation of reactive species that modify cellular DNA and protein; and/or chemotherapeutic properties, by forming derivatives that are antagonistic at the estrogen receptor (ER) or potentially antiangiogenic (7). Oxidative metabolism of estrogens, largely by hydroxylation, is mediated by the same CYPs that metabolize therapeutic agents and xenobiotics (15). Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidative metabolism of estrogens has been demonstrated for some, but not all, cytochrome P450. These CYPs include the major hepatic CYP 1A2 and 3A4 and the extrahepatic CYP 1A1 and 1B1 as well as members of the 3A family, which are expressed in a number of steroid hormone-responsive tissues, including brain, breast, ovary, kidney, and prostate (7,15). The biologic significance of tissue-specific expression of enzymes involved in extrahepatic estrogen metabolism remains largely unknown. However, it has been postulated by a number of investigators that local estrogen metabolism may generate important biologically active estrogen species or, in the

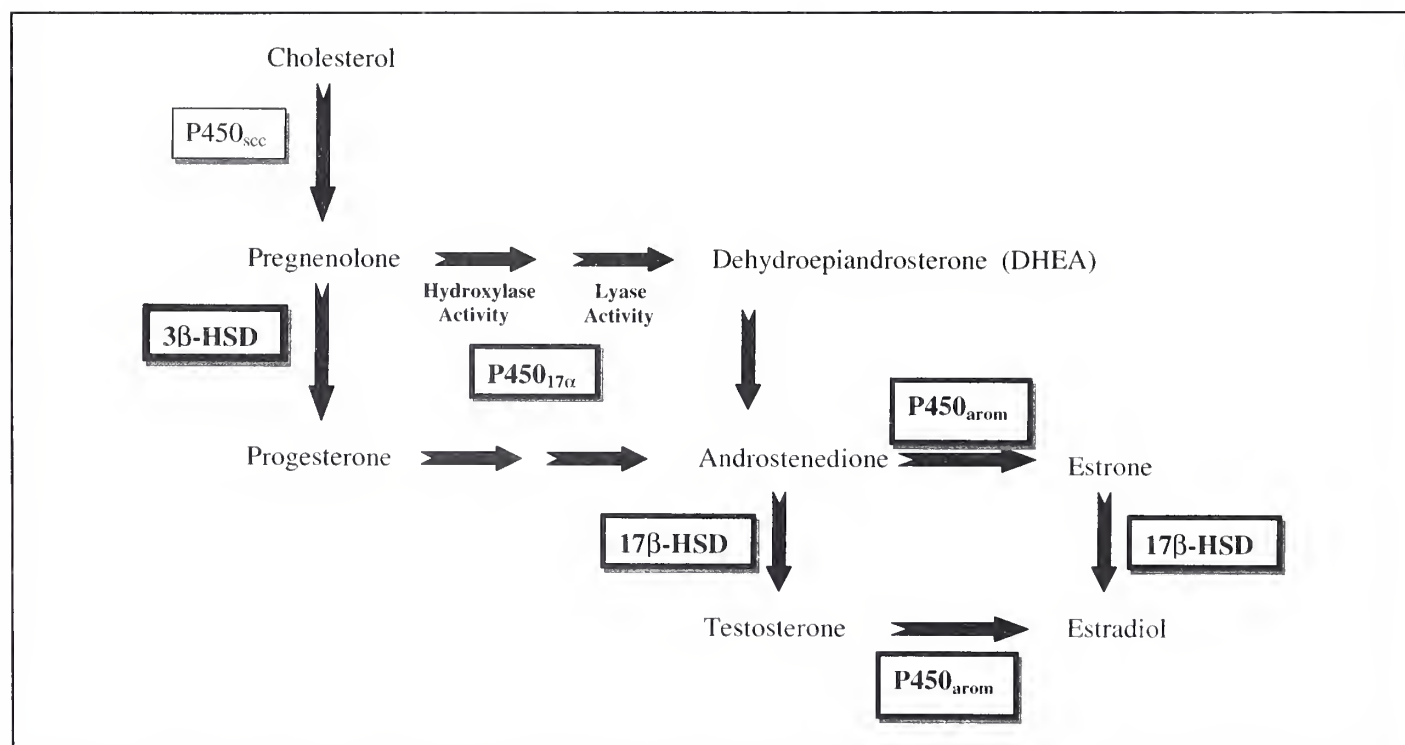


Fig. 1. Biosynthesis of estrogens from cholesterol. Participation of six key forms of cytochrome P450 and two hydroxysteroid dehydrogenases in the generation of estrogens are shown. The boxes containing enzymes with known polymorphisms are bolded.

case of carcinogenesis, may predispose certain tissues to exposures from highly reactive and genotoxic metabolites of E₂ [(7); Chapter 5].

Aromatic hydroxylation of E₂ occurs primarily at C-2 and to a lesser extent at C-4, to form the 2,3- and 3,4-catechol estrogens (CEs), respectively. These metabolites are intermediates for the generation of the reactive semiquinones and quinones. Both semiquinones and quinones have the ability to damage DNA and protein directly or indirectly through redox cycling and generation of reactive oxygen species (ROS), and they have been implicated as potential initiators of tumor formation (4,7,16,17). P450-mediated hydroxylation of estrone (E₁) at C-16 forms the "suspect" carcinogen 16 α -hydroxy E₁, an estrogen metabolite thought to bind covalently to DNA and ERs (18). Considerable controversy remains regarding which pathways and metabolites, if any, are most important in estrogen-induced carcinogenesis.

The secondary metabolism of E₂ involves *O*-methylation by catechol-*O*-methyltransferase (COMT), conjugation to glucuronides and sulfates, and clearance of reactive semiquinones and quinones, reported to involve catechol oxidation coupled to glutathione conjugation [(7); Chapter 6]. The disposition of the hydroxylated estrogens remains unclear and somewhat controversial. Conjugation of E₂ and E₁ to glucuronides and sulfates by specific enzymes in liver and target cells decreases their bioavailability by facilitating their excretion. However, there is increasing interest in these compounds as slow-clearing precursor compounds for biologically active estrogens reactivated, following cleavage of conjugated groups in target tissues (i.e., by sulfatases). Enzymatic *O*-methylation of CE by COMT leads to more lipophilic estrogen metabolites with longer half-lives than the other conjugated forms. As suggested by Zhu and Conney (7), studies indicate that these metabolites may have biologic activities atypical of those associated with classic ER occupation, some of which may be potential inhibitors of estrogen-dependent carcinogenesis. Because of the complexity of the biology of hormonal carcinogenesis and the experimental design issues for human population studies, we refer the reader to Chapters 3–5 and 8 for a comprehensive review of the current

competing hypotheses in estrogen-induced carcinogenesis and for the specific role of estrogen metabolites in breast carcinogenesis.

GENETIC POLYMORPHISMS IN ESTROGEN BIOTRANSFORMATION AND BREAST CANCER RISK

Many of the enzymes involved in estrogen metabolism/biosynthesis are polymorphically distributed within the human population (i.e., CYP17, CYP19, CYP3A4, CYP1A2, and COMT) (7,16,19,20), and, for some, there are known gene variants (i.e., CYP17, COMT, CYP1A1, CYP19, and glutathione *S*-transferase [GST]) that may account for a proportion of enzymatic variability (Table 1). For others with currently no defined genetic basis (e.g., CYP1A2 and CYP3A4) and, to some extent, for those with a defined variant allele, some portion of individual variability is determined by such factors as age, sex, smoking status, alcohol consumption, and dietary effects on the regulatory factors that control gene transcription and protein expression. Several research groups have begun measuring, in population-based studies, the distribution of some of the known allelic variants that have been predicted to alter estrogen metabolism and biosynthesis to determine the impact of these variants on breast cancer risk. Unlike measuring estrogen metabolites, an approach such as measuring genotype presents a more stable measure or biomarker of hormone exposure (i.e., cumulative high versus low), but it should be approached with caution because only a proportion, the extent of which is currently unknown, of individual variability in estrogen exposure may be explained by allelic variation. Investigating the distribution of functionally relevant genetic polymorphisms that alter the bioavailability of steroid hormones among people with disease and people without disease may provide more direct evidence for estrogen and estrogen metabolites as modifiers of human diseases, including breast cancer. The molecular epidemiologic studies and their results performed, to date, on genetic polymorphisms that alter estrogen metabolism/biosynthesis and breast cancer risk are reviewed below.

Table 1. Enzymes in the biosynthesis and metabolism of estrogen with known genetic polymorphisms analyzed to date in epidemiologic studies of breast cancer

	Role in estrogen biosynthesis and metabolism	Allelic variants/effect of genotype (reference No.)
CYP1A1	2-hydroxylase, extrahepatic (generates 2-OH CE)	M1, a Thr to Cys substitution 3' noncoding region; m2, an Ile to Val substitution in exon 7 (23); m3, an MspI RFLP that is specific to African-Americans (24); and m4, a Thr to Asp substitution in codon 461, which is adjacent to m2 (25)
CYP17	17 α hydroxylase/C17–20 lyase; catalyzes the rate-limiting step in the ovarian and adrenal biosynthesis pathways for androstenedione, the immediate precursor of testosterone	Base pair change in the 5'-untranslated region creates an Sp1-type (CCACC box) promoter site 34 bp upstream from the initiation but downstream from the transcription start site, predicted to introduce an additional Sp1 binding site and enhanced promoter activity (32)
CYP19	Aromatase/estrogen synthetase; converts testosterone and androstenedione to E ₂ and E ₁ , respectively	Polymorphic (TTTA) _n repeat in intron 5 close localization to the exon/intron border of exon 4, may alter splice site (43)
GST	Glutathione sulfotransferases; conjugate glutathione with reactive oxygen species decreasing oxidative stress generated during estrogen metabolism	GSTM1, deletion of the entire gene; ~50% of Caucasians inherit the null allele (60) GSTT1, deletion of the entire gene; ~30% of Caucasians inherit the null allele (65) GSTP1, Ile to Val change at amino acid position 105, reduced activity (30)
COMT	Methyltransferase; methylates and inactivates CE	Val to Met at amino acid position 158/108, alters heat stability, decreased methylation activity (47)
MnSOD	Manganese superoxide dismutase, mitochondrial superoxide dismutase that converts two superoxide radicals to H ₂ O ₂ and O ₂	Val to Ala change in the 9 amino acid position in signal peptide sequence, altered protein trafficking (59)

Ala = alanine; Asp = aspartic acid; Cys = cysteine; CE = catechol estrogen(s); COMT = catechol-*O*-methyltransferase; CYP = cytochrome P450; E₁ = estrone; E₂ = 17 β -estradiol; GST = glutathione-*S*-transferase; Ile = isoleucine; Met = methionine; RFLP = restriction fragment-length polymorphism; Thr = threonine; Val = valine.

CYP1A1

Early studies of genetic polymorphisms in CYP1A1 focused primarily on its role in lung cancer risk because it activates polycyclic aromatic hydrocarbons, potent tobacco smoke carcinogens. However, CYP1A1 also catalyzes the hydroxylation of E₂ at the C-2, C-6 α , and C-15 α positions in several extrahepatic tissues, including epithelial cells (15,21). To date, four polymorphisms have been identified within this gene; m1, which is a threonine to cysteine substitution that results in a *MspI* restriction site in the 3' noncoding region (22); m2, an amino acid substitution of isoleucine to valine in exon 7 of the gene (23); m3, an A-T to G-C transition mutation in the 3' noncoding region 300 base pairs (bp) from the polyadenylation site introducing an *MspI* restriction fragment-length polymorphism (RFLP) that is specific to African-Americans (24); and m4, an amino acid substitution of threonine to asparagine in codon 461 adjacent to m2 (25). Studies evaluating the role of CYP1A1 genotypes on breast cancer risk are summarized in Table 2.

Rebbeck et al. (26) investigated the m1 polymorphism in a small sample of case patients (n = 96) and "convenience sample" control subjects (n = 146). In the control subjects, the polymorphic allele had a low frequency (3% homozygotes and 6% heterozygotes) that was similar to that observed among the case patients. In the Western New York Diet Study, Ambrosone et al. (27) evaluated the m2 polymorphism in a case-control study with 404 postmenopausal women. The exon 7 substitution was associated with a nonsignificant increase in risk that was most pronounced among women who were moderate smokers (<20 pack years). Taioli et al. (28) hypothesized that CYP1A1 polymorphisms could affect breast cancer risk through their mediating effect on estrogen metabolism. In a case-control study of African-American and European-American women (51 case patients and 269 control subjects), they noted that, among African-American women, the m1 polymorphism significantly increased breast cancer risk (odds ratio [OR] = 9.7; confidence interval [CI], 2.0–47.9). Numbers in these stratified analyses, however,

were quite small. With the use of data from the Nurses' Health Study, Ishibe et al. (29) studied the effects of CYP1A1 polymorphisms (m1 and m2) and cigarette smoking on breast cancer risk in a European-American population. Although women with variant alleles for either polymorphism were not at increased risk overall, those women with variant alleles who began smoking before the age of 16 years were at significantly increased risk of breast cancer. Recently, Bailey et al. (30) evaluated all four known CYP1A1 polymorphisms in relation to breast cancer risk in a case-control study with approximately 164 Caucasian and 59 African-American women with breast cancer and equal numbers of age-matched control subjects. None of these polymorphisms, including those specific to African-Americans, was associated with increased risk; risk was not modified by smoking status "ever/never."

CYP17

Another cytochrome P450 enzyme that has received much attention of late is CYP17. As discussed above, CYP17 functions at key branch points in human steroidogenesis, catalyzing the ovarian and adrenal biosynthesis pathways for androstenedione, the immediate precursor of testosterone (Fig. 1) (31). A single base-pair change in the upstream promoter site creates an additional *MspI* recognition site in the 5'-untranslated region and creates a Sp1-type (CCACC box) promoter site 34-bp upstream from the initiation, but downstream from the transcription start site. This variant is referred to as the A2 allele and is predicted to introduce an additional Sp1 binding site and enhanced promoter activity (32).

Feigelson et al. (32) hypothesized that the polymorphism (A2 allele) could result in an increased rate of transcription and, thus, an increase in estrogen levels and perhaps increased risk of breast cancer in carriers of the variant allele. In a multiethnic cohort (Latino, Asian, and African-American) in Los Angeles and Hawaii, the researchers genotyped DNA from 174 case patients and 285 control subjects. Allele differences were not different between groups, so all were analyzed together. Overall, risk was not significantly increased for women with the A2 allele, but when women were stratified by stage of disease, it was observed that the A2 allele conferred more than a twofold

Table 2. Associations between breast cancer and CYP1A1 variant alleles*

Study (reference No.)	Polymorphism	Case patients	Control subjects	OR (95% CI)	Modifying factor	OR (95% CI)
Rebbeck et al., 1994 (26)	m1	96	146	Not calculated, no association		
Ambrosone et al., 1995 (27)	m2 (heterozygote & homozygote)	176	228	1.61 (0.9–2.8)	Smoking, <20 pack yr	5.2 (1.2–23.7)
Taioli et al., 1995 (28)	m1	51	269		African-American women only (20 case patients, 81 control subjects)	9.7 (2.0–47.9)
Ishibe et al., 1998 (29)	m1	466	466	1.5 (0.7–1.5)	Smoking	5.7 (1.5–21.3)
	m2			0.9 (0.6–1.3)	Smoking < age 16 yr	3.6 (1.1–11.7)
Bailey et al., 1998 (30)	m1	164, Caucasian	162, Caucasian	1.4 (0.8–2.4)		
	m2			1.4 (0.6–3.1)		
	m3			—		
	m4			0.8 (0.4–1.9)		
	m1	59, African-American	59, African-American	0.5 (0.2–1.1)		
	m2			1.0 (0.98–1.1)		
	m3			0.8 (0.3–2.0)		
	m4			1.0 (1.0–1.1)		

*OR = odds ratio; CI = confidence interval.

increase in risk among those with advanced disease. The researchers also noted that late age at menarche was protective only among women who were homozygous for the A1 allele. Because it is unknown whether or not this polymorphism is functional, Feigelson et al. (33) followed this report with a controlled study of serum hormone levels throughout the menstrual cycle in nulliparous healthy women. Among women with A2 alleles, levels of E_2 and progesterone were consistently higher on days 11 and 22, respectively. Further studies, however, have failed to confirm these findings.

In a large case-control study in the U.K. (835 case patients and 591 control subjects), Dunning et al. (34) found no evidence of increased risk for women with variant alleles. CYP17 did not modify associations between age at menarche and risk and there was no effect observed among women with advanced disease. Similar null associations were noted in studies by Helzlsouer et al. (35) and by Weston et al. (36), and our own analyses in the Western New York Diet Study (unpublished data) also support the null hypothesis of no association between CYP17 genotype and breast cancer risk. Most recently, Haiman et al. (37) assessed the association between the A2 variant of CYP17 and breast cancer risk in a prospective nested case-control study in the Nurses' Health Study Cohort. Within the Nurses' Health Study, women with the A2 allele were not at increased risk for incident breast cancer or for advanced disease. However, like the findings of Feigelson et al. (32), the protective effect of later age at menarche (>13 years) was only observed among women with the A1 allele and not among women carrying the A2 alleles, adding further support to the hypothesis that the A2 variant allele in CYP17 may act as a modifier of breast cancer risk but is not an independent risk factor.

CYP19

Aromatase or estrogen synthetase, encoded by the CYP19 gene, converts androstenedione to E_1 and testosterone to E_2 and completes the pathway for estrogen biosynthesis from cholesterol (38). The majority of circulating estrogens in premenopausal women is in the form of E_2 and is produced cyclically by the granulosa cells of the ovarian follicles (39). However, extensive extragonadal production of estrogens also occurs in liver, muscle, and adipose tissue by aromatization of adrenal androgens (40). After menopause, the majority of estrogen is derived from fat by the aromatization of adrenal androstenedione to E_1 , a weaker estrogen than E_2 . The extent of androstenedione conversion to E_1 is associated with higher body fat content and with increasing age, indicating that a major source of estrogen exposure in older women is the continuous production of E_1 in adipose tissue (41). Several studies [reviewed in (42)] have suggested a relationship between increased adiposity, elevated E_1 levels, and postmenopausal breast cancer risk.

A polymorphic tetranucleotide repeat (TTTA)_n has been identified in intron 5 about 80 nucleotides downstream of exon 4 in the CYP19 gene near the intron/exon border. This close proximity to the intron/exon suggests a possible role for these tetranucleotide repeats in the determination of splicing sites. In a study performed by Kristensen et al. (43), five different alleles containing 7, 8, 9, 11, and 12 TTTA repeats were identified. Although relatively rare, Kristensen et al. (43) noted a significant association with breast cancer risk in carriers of the longest repeat variant (TTTA)₁₂, designated the A1 allele, in a case-control study with 366 case patients and 252 control sub-

jects. The A1 allele was present in less than 2% of the control population but in almost 4% of case patients. Siegelmann-Danieli et al. (44) also evaluated this association and found increased risk with the variant A1 allele. These data suggest that polymorphisms in the CYP19 gene may be involved as a low-penetrance gene in breast cancer susceptibility. Other polymorphisms have been identified in the coding region of CYP19, but they do not appear to alter enzyme function or expression (45) and have not been analyzed in population studies in association with risk.

COMT

COMT is one of several phase II enzymes involved in the conjugation and inactivation of CE (46). Because there is evidence that CE, particularly the 4-hydroxyCE, may bind to DNA and result in DNA damage (4,17), the possible role of lower activity in the enzyme in relation to breast cancer risk is important. An amino acid change (valine to methionine) at position 158/108 in the membrane-bound/cytosolic form of the protein has been linked to decreased methylation activity of COMT (47). The allelic variation at amino acid position 158/108 is believed to be closely associated with the observed trimodal distribution of COMT enzyme activity in the human population. The genotypes designated in relation to the predicted enzymatic activity of the protein are high (COMT^{Val/Val}), intermediate (COMT^{Val/Met}), and low (COMT^{Met/Met}) (48,49). Three groups to date, all with conflicting results (Table 3), have evaluated the role of the COMT genetic polymorphism in relation to breast cancer risk.

In a nested case-control study, Lavigne et al. (50) evaluated COMT low-activity alleles in 113 women with breast cancer and an equal number of control subjects. In the entire sample, overall associations with heterozygosity and homozygosity for the "low-activity allele" were 1.30 (95% CI, 0.66–2.58) and 1.45 (95% CI, 0.69–2.58), respectively. When women were stratified by menopausal status, women who were postmenopausal had a greater than twofold increase in risk with the COMT^{Met/Met} genotype or two low-activity alleles, but inverse associations were noted for premenopausal women with the same genotype. Because in postmenopausal women, most estrogens are produced by the conversion of androgens in adipose tissue, associations were also evaluated among women stratified on body mass index (BMI). Among postmenopausal women, associations were noted only among those whose BMI was greater than 24.47 kg/m².

Thompson et al. (51) performed similar analyses in a study of 281 case patients and 289 control subjects in western New York. In the overall dataset, no relationship was observed between variant COMT alleles and breast cancer risk, but marked differences were noted when data were stratified by menopausal status. Among premenopausal women with breast cancer, those with at least one low-activity allele showed significantly increased risk (OR = 2.4; 95% CI = 1.4–4.3). In contrast to premenopausal women, there was an inverse association between low-activity alleles and postmenopausal breast cancer, which was most pronounced among those who were COMT^{Met/Met} (OR = 0.4; 95% CI = 0.2–0.7). When COMT^{Met/Val} individuals were combined with individuals who were COMT^{Met/Met}, having one or two low-activity alleles significantly decreased risk (OR = 0.5; 95% CI = 0.3–0.9). When data were stratified by menopausal status, the low-activity

Table 3. Associations of a low-activity allelic variant of COMT with breast cancer risk according to menopausal status*

Study (reference No.)	Study size case patients/control subjects	COMT low-activity allele alone	OR (95% CI)	Interaction	Exposure
Lavigne et al., 1997 (50)					
All subjects	113/114	No association with breast cancer risk	1.4 (0.7–2.9)		
Premenopausal	24/25	Decreased risk of breast cancer	0.2 (0.04–1.5)		
Postmenopausal	89/89	Increased risk of breast cancer	2.2 (0.9–5.1)	Highest risk for breast cancer in women with BMI >24.47 g/m ² ; OR = 3.6 (95% CI = 1.1–12)	
Thompson et al., 1998 (51)					
All subjects	281/289	No association with breast cancer risk			
Premenopausal	141/134	Increased risk of breast cancer	2.1 (1.0–4.4)	Highest risk for breast cancer strongest in heaviest women with BMI >27 kg/m ² ; OR = 5.7 (95% CI = 1.1–30.1)	Increased risk for breast cancer only among ever smokers
Postmenopausal	140/155	Decreased risk of breast cancer	0.4 (0.2–0.7)	Decreased risk for breast cancer strongest in leanest women with BMI <23 kg/m ² ; OR = 0.3 (95% CI = 0.1–0.7)	Decreased risk only significant among never smokers
Millikan et al., 1998 (54)					
All subjects	654/642	No association with breast cancer risk			
European-American	389/379	No association with breast cancer risk	0.7 (0.5–1.1)		
African-American	265/263	No association with breast cancer risk	0.8 (0.4–.5)		
Premenopausal (combined European-American and African-American)	331/297	No association with breast cancer risk; RR = 0.7 (0.4–1.2)		Decreased risk for breast cancer in heaviest women with BMI >27.8 kg/m ² ; RR = 0.5 (0.3–1.1)	Decreased risk for breast cancer in women who were physically inactive; RR = 0.5 (95% CI = 0.2–0.9)
Postmenopausal (combined European-American and African-American)	323/344	No association with breast cancer risk; RR = 0.8 (0.5–1.4)			Decreased risk for breast cancer in women who were physically inactive; RR = 0.5 (95% CI = 0.3–0.9)

*BMI = body mass index; CI = confidence interval; COMT = catechol-O-methyltransferase; OR = odds ratio; RR = relative risk.

COMT^{Met} variant was most strongly associated with risk among the heaviest premenopausal women (OR = 5.7; 95% CI = 1.1–30.1); whereas, in postmenopausal women, an inverse association with COMT and risk was strongest in the leanest women with at least one low-activity allele (OR = 0.3; 95% CI = 0.1–0.7). The authors hypothesized that there may be an opposing role of CE metabolism in breast cancer etiology, depending on the hormonal environment, and that differing biologic effects of the CE reported in the literature (i.e., DNA damaging versus growth inhibition) may depend on the levels of circulating estrogens. They further suggested that, in a high-estrogen environment, such as in premenopausal and the heaviest postmenopausal women, higher circulating levels of the catechol compounds (2-OH and 4-OH) may result in higher circulating levels of potentially mutagenic compounds (17). In a low-estrogen environment, as in leaner postmenopausal women, higher circulating levels of the unmethylated catechols in a low COMT background may elevate the levels of the putative anticarcinogenic 2-OHE₁ (52).

Because the CEs are products of estrogen metabolism by CYP1A1 and CYP1A2, which are both induced by smoking, Ambrosone et al. (53) have also presented data evaluating the role of COMT on breast cancer in smoking and nonsmoking women. It is interesting that increased risk was observed only among premenopausal women who smoked and that inverse

associations were significant only among postmenopausal non-smokers. Millikan et al. (54) also evaluated these possible relationships in the Carolina Breast Cancer Study (654 case patients and 642 control subjects), within a population of European-American and African-American women. Neither low- nor high-activity alleles were associated with increased breast cancer risk for premenopausal or postmenopausal women, European-American women, or African-American women. Among premenopausal women (European-American and African-American women combined), there were inverse associations between low-activity alleles and breast cancer among women with BMI greater than 27.8 and among both premenopausal and postmenopausal women who were physically inactive. Smoking, hormone replacement therapy, and oral contraceptive use did not modify associations. These discrepancies may be due to small sample sizes in the studies by Lavigne et al. (50) and Thompson et al. (51), or there may be biologic factors that differentially impact risk associations; these issues will be addressed below.

GST and MnSOD

ROS may be generated through a number of mechanisms, including those related to metabolism of E₂ (see Chapters 4 and 5). For example, ROS are produced via CE-mediated redox cycling of quinones and semiquinones (16). Substantial data indicate that oxidative stress is related to breast cancer risk (55–57).

The glutathione-dependent peroxidases (e.g., GST and selenium-dependent glutathione peroxidases) are involved in detoxification of products of oxidative damage, by catalyzing conjugation of glutathione with ROS (58). Similarly, superoxide dismutase (Mn, Cu, and ZnSOD) catalyzes the dismutation of two superoxide radicals, producing hydrogen peroxide and oxygen. Genetic polymorphisms are known to affect enzyme activity in GST M1, T1, and P1, and a recently identified polymorphism in MnSOD apparently alters the structure of the enzyme, affecting its ability to enter the mitochondrion (59). Polymorphisms in these enzymes could impact the relationship between oxidative stress and breast cancer etiology.

The GSTM1 genetic polymorphism is a deletion of the entire gene; approximately 50% of European-Americans inherit the null allele (60). Because GSTM1 enzyme is present in human breast tissue (61), it is plausible that lack of this isozyme could increase breast cancer risk. A number of research groups have evaluated possible associations between GSTM1 and breast cancer risk and, for the most part, have found no effect on risk (see Table 3). In a study of 197 women with breast cancer and 225 control subjects, Zhong et al. (62) found no increased risk with the null allele. Similarly, Ambrosone et al. (27,63) found no association between GSTM1 genetic polymorphisms and breast cancer in a study with 212 premenopausal and 410 postmenopausal women. Risk relationships were not affected by smoking status or by high or low consumption of dietary sources of antioxidants. In the Nurses' Health Study, Kelsey et al. (64) also observed no overall associations between the GSTM1 null allele and breast cancer risk; however, the deletion appeared to be associated with better survival, perhaps because of the role of GST in metabolism of and conjugation with chemotherapeutic agents. Supporting these negative findings, in a study with 164 European-American and 59 African-American women with breast cancer and an equal number of matched control subjects, Bailey et al. (30) also found no associations between GSTM1 genotype and breast cancer risk. Associations were observed, however, with not only GSTM1 polymorphisms but also with GSTT1 and GSTP1, in a nested case-control study evaluated by Lavigne et al. (50) for COMT. Helzlsouer et al. (65) noted that the null genotype was associated with a twofold increase in breast cancer risk, primarily among postmenopausal women. Although not significant, the GSTP1 polymorphism also appeared to increase risk. When combining putative "high-risk" alleles for the three genes, women who were null for GSTT1 and GSTM1, and heterozygous or homozygous for the GSTP1 valine substitution, had an almost fourfold increase in breast cancer risk (OR = 3.77; 95% CI = 1.10–12.88). Bailey et al. (30) also evaluated GSTT1 in relation to breast cancer in the previously noted study, but observed no association with increased risk. More recently, Garcia-Closas et al. (66) reported no evidence for an association between GSTT1 null (OR = 0.86; 95% CI = 0.61–1.21) or GSTM1 null (OR = 1.05; 95% CI = 0.80–1.37) in 466 women with incident breast cancer compared with an equal number of control subjects in the Nurses' Health Study. Furthermore, when GST genotypes were considered in combination or together with cigarette smoking, no associations with an increased risk of breast cancer were observed.

Until recently, the MnSOD polymorphism had been evaluated only in relation to Parkinson's disease in a study in Japan (59). Because ROS, including those generated by estrogens and their metabolites (Chapter 4), may be involved in breast carci-

nogenesis and because MnSOD is a major enzyme involved in the scavenging of free radicals, Ambrosone et al. (67) hypothesized that the MnSOD alanine allele could be related to breast cancer risk by having an altered capacity to reduce oxidative stress. In the western New York study, the variant allele was associated with an almost twofold increase in risk in postmenopausal women and, in premenopausal women, heterozygosity or homozygosity for the alanine allele conferred a 3.5 risk (95% CI = 1.8–6.8). Risk appeared to be the greatest among women who were in the lower median for consumption of dietary sources of antioxidants, fruits, and vegetables overall.

METHODOLOGIC ISSUES IN MOLECULAR EPIDEMIOLOGY

The molecular epidemiologic study of susceptibility inferred by genetic polymorphisms and the elucidation of gene-environment interactions should advance our understanding of carcinogenic mechanisms. But many studies of polymorphisms in xenobiotic and steroid hormone-metabolizing enzymes and cancer risk, and those of gene-environment interactions have yielded conflicting results. The molecular epidemiologic literature is rife with inconclusive data. There is a clear need for the molecular epidemiologic community to explore areas of bias and flaws in study design and analyses that may result in inconsistent study results.

A number of plausible explanations are available for conflicting results in gene-environment interaction studies, the most obvious of which would be related to small sample size and misclassification of exposure, which can result in both Type I and Type II errors. A Type II error, which represents the inability to detect a true effect, results from inadequate power. Statistical power depends on sample size, the size of the effect to be detected, and the variability within the study population. Small sample sizes are common to molecular epidemiologic studies, not only because population-based molecular studies are expensive and, thus, restrict the number of subjects recruited but also because the means of analysis automatically cut the population in half or, in many instances, well below that in stratified analyses. For studies of gene-environment interaction, case and control subjects are stratified by genotype, and associations between the risk factor and disease status are evaluated separately within each stratum. Theoretically, one would stratify by genotype and calculate relative risks or ORs to estimate risk associated with an exposure, adjusting for possible confounding factors or other known disease risk factors. Clearly, even in large studies, the numbers of subjects in each cell will be drastically reduced, greatly decreasing power and the likelihood of being able to detect an effect. Less often considered, however, is the likelihood of a Type I error (i.e., a false-positive result). As pointed out by Greenland and Rothman (68), when stratification has exceeded the limits of the data, the exposure effect estimates begin to get further and further from the null, and the OR becomes inflated. Bias in the analysis may be introduced in the process of applying large-sample methods to sparse data. Additionally, even modest errors in exposure measurements or genetic tests can confer a significant bias in epidemiologic studies, requiring even larger-than-expected sample size to compensate for misclassification (69). As recently demonstrated and discussed by Garcia-Closas et al. (69), the importance for improved accuracy and validation of exposure assessment and establishment of good quality control for genetic testing should be con-

sidered areas of high priority in gene-environment interaction studies.

However, despite issues related to small sample sizes, results may also vary because of the nature of the individual study designs and the populations being studied. For the most part, investigators have used three approaches to study gene-environment interactions: 1) to evaluate effects of a polymorphism alone on cancer risk, 2) to investigate the effects of the polymorphism on risk among persons exposed and unexposed to an environmental factor, and 3) to examine the effects of an exposure on risk within individuals with varying genotypes. This approach however, is inherently limited by the amount of information that genotype confers on enzyme activity. Genotype alone is an inexact or incomplete surrogate for the knowledge of enzyme activity that one would want and does not take into account the induction or inhibition of enzyme activity. And, other than a handful of null and inactive allelic variants (i.e., GST M1 and T1), genotype alone explains only a proportion of the phenotype. Levels of enzyme activity may rise or decline depending on the effects of any number of endogenous and exogenous factors. For example, enzyme induction may occur through the effects of steroid hormones or by exposure to drugs, pesticides, industrial chemicals, tobacco smoke, ethanol, and food sources. Conversely, enzyme activity can be inhibited through competitive binding at the active site, decreased biosynthesis, or increased breakdown of the enzyme by a number of chemicals and foods.

An example of the impact of these multiple variables on predicted associations is demonstrated in the unexpected observation that GST M1 may increase the risk of colorectal adenomas in a diet high in broccoli (70). The mechanism proposed by these investigators was that the GSTs conjugate the cancer chemopreventive isothiocyanate, sulforaphane, rendering it inactive. Thus, although one would intuitively presume that an absence of GST would increase cancer risk through a decreased ability to conjugate with ROS and chemical carcinogens as discussed above, lack of GST may actually be protective by not negating the anticarcinogenic effects of isothiocyanates. Because of the many factors that can impact enzyme activity, inconsistencies in results of studies measuring genotype as an independent marker of enzyme activity could be related to differential exposures of study populations to known or unknown factors that affect enzyme levels.

Thus, molecular epidemiologists face formidable challenges in study design to investigate accurately the role of hormone metabolites in breast cancer risk, particularly through assessment of genetic polymorphisms in enzymes involved in hormone metabolism. First, a strong understanding of biologic mechanism of disease will be essential in defining and accurately monitoring risk-related exposures in subsets of people defined by genetic sensitivity. Second, large studies are needed, as well as creative analytic approaches, to be able to evaluate accurately the effects of multiple genes and multiple exposures on breast cancer risk.

FUTURE DIRECTIONS

The first approach to be applied to the study of susceptibility to breast cancer would be a reassessment of breast cancer disease and the related risk factors. Several intriguing but unconfirmed observations suggest that tumor phenotypes clinically subtyped by (ER)/progesterone receptor (PR) status, p53 mutations, or

HER-2/neu may not only be valuable as prognostic indicators for patient outcome but may also reflect distinct etiologic pathways, providing a means to mirror past exposures (71-75). For example, breast tumors that overexpress the oncogene HER-2/neu have been suggested to represent a breast cancer subtype with a common etiologic origin that may have evolved from a pathway separate from other breast cancers (74,76). The hypothesis that separate breast tumor subtypes exist has led to the corollary that these tumor subsets may have distinctive sets of risk factors relevant to differing causative events. This concept merits serious consideration in future study designs. For example, amplification of the oncogene HER-2/neu, associated with a poor prognostic tumor phenotype, has been positively associated with early use of oral contraceptives (71,72) and inversely associated with breast-feeding (73,74). The overexpression of p53 has been positively associated with current smoking (77) and with oral contraceptive usage (78). In the Iowa Women's Health Study, Potter et al. (75) evaluated classic epidemiologic risk factor data to determine the interaction between breast cancer risk factors (i.e., family history, BMI, reproductive factors, hormone use, and alcohol use) and ER/PR status. In this study of 939 incident breast cancers, three patterns of association appeared in relation to epidemiologic risk factors. PR-positive breast cancers were associated with endogenous hormone exposure variables, whereas ER-negative/PR-negative and ER-positive/PR-positive breast cancers were inversely associated with a number of the widely accepted reproductive risks. Although the data are not presented but referred to, Huang et al. (79) also indicate significant differences for reproductive risk factors among case subjects stratified by ER/PR status in the Carolina Breast Cancer Study. These observations suggest that tumor phenotypes subtyped by ER/PR status, HER-2/neu, or p53 may reflect distinct clinical entities. Their etiologies may be related to host environmental and lifestyle factors that ultimately impact the natural history of the disease. Overall, these data are provocative and suggest that etiologic heterogeneity in subsets of breast cancers may have masked the impact of relevant risk factors. Incorporation of tumor phenotyping in disease stratification may yield stronger and more consistent associations with etiologic and genetic factors, such as reproductive variables, smoking, exogenous hormone usage, and metabolic variability among subsets of breast tumors.

A second important area for future studies is the continued identification of functionally significant gene polymorphisms and the incorporation of research platforms to rapidly interrogate the associations between allele variability, exposure, and risk. Unfortunately, the identification of functionally distinct genetic variants that alter activity or gene expression in a modest fashion (i.e., changes of activity or expression), as opposed to rare mutations that result in overt metabolic deficiencies, is difficult and has been slow. The identification of widely distributed mutations or polymorphisms has been greatly facilitated by the ongoing efforts to map the more than 80 000 genes in man. This explosion in information on the genetic heterogeneity of man will be fundamental to understanding complex disease risk. However, the incorporation of sequence-based information in population studies will only be practical with the validation of high-throughput, DNA-based methodologies, as promised by a number of newly emerging platforms, such as DNA microarrays, multiplex analysis on fluorescent microspheres, or multiplex mass-tag strategies (80). The introduction of technologies that

provide simultaneous assessment of tens to hundreds of allelic variants in large numbers of samples promises rapid, accurate, and cost-effective iterative hypothesis testing for gene-based complex diseases.

With the tools to more clearly understand cancer etiology and to identify susceptible subgroups of individuals also comes the responsibility to devise strategies to produce results that are valid. Because of the pitfalls and sources of bias in molecular epidemiologic studies, it is imperative that we seriously critique our own data, establish working consortia to develop and to capitalize on large cohort studies, and introduce efforts to increase public and professional awareness of the need to perform human population studies. Presentation of data from small studies, even though they may conflict with each other, will move the field forward. As insights are gained from epidemiologic studies and taken to the laboratory, they will help us to understand mechanisms of carcinogenesis and should be encouraged but interpreted with caution. At present, this remains an area of fundamental research with potential to significantly impact our understanding of individual variability and disease risk within the human population. Alternatively, this research should provide much needed information for identifying subpopulations that would most benefit from aggressive prevention strategies.

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Chapter 8: Estrogen Receptor-Mediated Processes in Normal and Cancer Cells

Robert B. Dickson, George M. Stancel

The role of estrogens in breast and other cancers has been extensively investigated for many years, and historically most of these studies have focused on the hormonal regulation of cell proliferation. The most recent work in this area has focused on the expression of genes likely to mediate proliferation (e.g., growth factors, proto-oncogenes, etc.) and their regulation by the classic nuclear estrogen receptor, ER- α . In this chapter, we present a synopsis of several new developments in this area of ER-regulated gene expression. These developments include the following: 1) the selective activation of ER domains by partial estrogen antagonists, such as tamoxifen and other ligands; 2) the effects of ER- α overexpression and gene knockout on the development of breast and uterine cancers in experimental animal models; 3) mechanisms by which steroid hormones regulate programmed cell death, cell cycle progression, cell-substratum interactions, and genomic instability in cancer cells; 4) identification of nuclear proteins that interact with the ER in the presence of agonists and antagonists, the effect of ligand binding on the receptor structure, and the interactions of liganded and nonliganded receptors with coactivators, corepressors, and other regulatory proteins; and 5) the biochemical properties, cellular distribution, and potential biologic roles for the newly discovered ER- β . Although there is an increasing interest in understanding the role of estrogens as endogenous carcinogens, it remains clear that ER-mediated regulation of gene expression plays many significant roles in normal and cancer cells, and increased knowledge of the mechanisms involved will improve our overall understanding of hormonal carcinogenesis. [J Natl Cancer Inst Monogr 2000;27:135–45]

BACKGROUND AND RATIONALE FOR SESSION

The primary focus of this meeting was “Estrogens as Endogenous Carcinogens in the Breast and Prostate,” and most speakers thus discussed the actions of estrogens that are potentially related to initiation events (Chapters 3–5). This focus is somewhat different from most historic studies on estrogens and cancer, which focused largely on the role of estrogens in the process of proliferation. The view was that estrogens increased proliferation of target cells in the breast and other tissues, and this proliferation contributed to breast cancer by one of two major mechanisms. First, an increase in cell proliferation would be expected to cause an increase in spontaneous errors associated with DNA replication. Second, after mutations were introduced into a target cell by this or other mechanisms, estrogens would enhance the replication of clones of cells carrying such genetic errors. Much of the focus on estrogens in cancer to date has thus been on the mechanisms by which estrogens increase cell proliferation.

The general view has been that estrogens regulate proliferation of target cells by transcriptional mechanisms involving the

classic estrogen receptor (ER), initially discovered in the laboratories of Elwood Jensen at the University of Chicago, IL, and Jack Gorski at the University of Illinois (Champaign-Urbana). Work from their laboratories, along with metabolic inhibitor studies of Gerald Mueller, then at the University of Wisconsin (Madison), led to the concept that estrogens increased proliferation by stimulating RNA synthesis in target cells. This concept led to a search for target genes for which transcription was regulated by estrogenic hormones, and many laboratories identified a number of growth factors, proto-oncogenes, and other regulatory molecules that were likely candidates for such genes.

In recent years the emphasis of these studies has progressed to investigating the transcriptional regulation of such target genes by the ER, with special emphasis on identifying the regulatory factors involved and their molecular mechanism of action, differences in the activity of various ER ligands, the identification of new ER subtypes, and the types of gene families regulated by estrogens during hormonally induced increases in proliferation. It was thus felt important to have a session on ER-mediated processes in normal and target cells. Because it was impossible to present a comprehensive review of this body of work in a single session, the goal was to invite a group of speakers who would address some of the most rapidly emerging paradigms of estrogen action that the Organizing Committee felt were particularly relevant to understanding the actions of estrogens in cancer cells.

Readers interested in additional information on this aspect of estrogen action are referred to a number of recent reviews and articles and references therein, in addition to the references provided throughout the body of this article. These references include information on the structure and function of the nuclear ERs (1–9), the roles of nuclear receptor coactivators and corepressors in steroid hormone action (10–12), recent advances in the development of selective ER modulator(s) (SERM) (13–17), and the various phenotypes observed in ER knockout mice, which indicate biologic actions mediated by these nuclear receptors (18–21).

OVERVIEW OF SPEAKERS AND TOPICS

Until quite recently, the stimulation of transcription by estrogens was viewed in terms of a relatively straightforward set of interactions initiated by estrogen binding to a single type of ER that was thought to be identical in all target tissues. Ligand

Affiliations of authors: R. B. Dickson, Lombardi Cancer Research Center, Georgetown University, Washington, D.C.; G. M. Stancel, Department of Integrative Biology, Pharmacology, and Physiology, University of Texas Medical School at Houston.

Correspondence to: George M. Stancel, Ph.D., Department of Integrative Biology, Pharmacology, and Physiology, University of Texas Medical School at Houston, 6431 Fannin, Houston, TX 77030.

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binding was viewed as a "trigger" that activated the receptor from an "off" state to an "on" state, and this activation enabled the receptor to activate or repress transcription of target genes. The activated receptor was then thought to interact with an estrogen response element (ERE) in the 5'-flanking region of responsive genes. It was thought that EREs of most endogenous hormone-regulated genes would have sequences similar to the palindromic sequence, GGTCAnnnTGACC, originally identified in the vitellogenin gene and generally referred to as the consensus ERE. This scheme of estrogen action is illustrated in Fig. 1, and, although highly schematized and oversimplified, it represents, to a good approximation, the state of our basic knowledge of estrogen-regulated transcription about 5 years ago.

In terms of cancer, it has been known for many years that breast cancer cells contain steroid receptors, and the content of ERs and progesterone receptors (PRs) in individual tumors is a valuable predictor of whether an individual patient will respond to endocrine therapy. However, the correlations between receptor content and responses to endocrine therapy are far from perfect, and many tumors progress to states of hormone independence. Antihormones, such as tamoxifen, were known to compete with estrogenic agonists for receptor binding, but little else was known about the specific biochemical mechanisms by which these important drugs produced their actions in experimental or therapeutic settings. In addition, their use is complicated because most breast tumors eventually become refractory to antiestrogen treatment. Paradoxically, drugs such as tamoxifen also display strong agonist activity in the endometrium, which is highly problematic for their therapeutic use. Such observations were difficult to reconcile with a view of the ER as a simple "on/off" switch that interacted with the same regulatory sequence in all target genes.

Within recent years, significant advances in our understanding of ER-mediated events have occurred at the conceptual level, and major new experimental approaches to the study of hormone action have become available. Many of these approaches will be discussed in this session, and several key points are enumerated below.

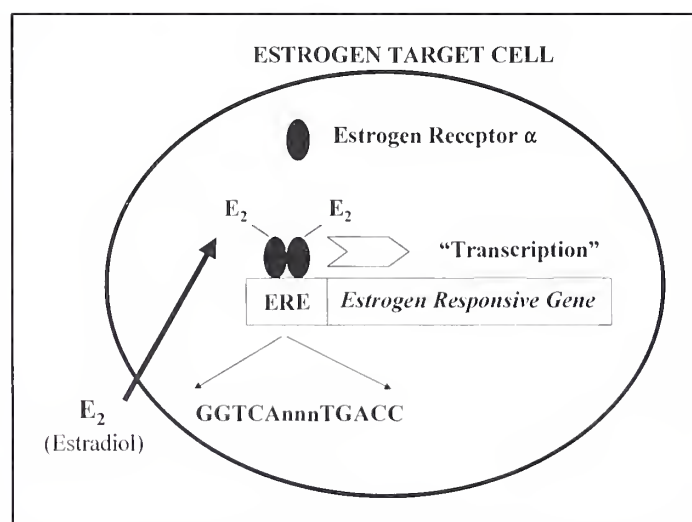


Fig. 1. Model of estrogen action circa 1990. Estrogens such as estradiol (E_2) enter target cells by diffusion and bind the classic estrogen receptor- α (filled ovals). The receptor-hormone complex stimulates transcription of target genes via interactions with an estrogen response element similar to the sequence 5'-GGTCAnnnTGACC-3' identified in the vitellogenin A2 gene.

- 1) It is now known that the ER contains several "domains" that are involved in transcriptional regulation and that different ligands may selectively activate these functions. This knowledge raises the exciting prospect of developing estrogens that can be used to selectively produce desired therapeutic actions while minimizing untoward side effects; several such agents have already been discovered. Such functional studies on the actions of different estrogens and antiestrogens are being accompanied by structural studies of the molecular interactions between ligands and the receptor, and this combination will almost certainly lead to the discovery of even more selective agents. A related question of special importance to understand breast cancer etiology is whether structurally diverse estrogens differentially stimulate proliferation of breast cancer cells. Dr. McDonnell discusses the role of the ER transcriptional activation functions (AFs) in ligand selective responses.
- 2) A major experimental advance has been the production of experimental animals that overexpress the ER or knockout animals that do *not* express the receptor. These experimental animals provide heretofore unavailable approaches to define unequivocally the role of ER in estrogen-mediated events, to identify redundant signaling pathways that compensate for changes in ER levels, and to identify previously unrecognized actions of estrogens. Dr. Couse describes the generation and phenotypes of ER- α knockout (ER α KO) and overexpressing mice as well as the effect that receptor levels have on the development of breast and uterine cancers in experimental models.
- 3) In the past, a major focus of study has been the regulation of growth factor and proto-oncogene expression. More recently, attention has increased to other ways by which estrogens might affect breast cancer. This attention includes the study of mechanisms that regulate cell death, the factors that control cell-cycle progression, and the mechanisms that contribute to genomic instability of cancer cells. In addition, interest has increased in processes, such as angiogenesis and cell-substratum interactions, that can affect tumor growth and metastases, and understanding these processes may also improve our understanding of the etiology of breast cancer and potential therapeutic targets. Dr. Dickson addresses several mechanisms regulating these pathways in mammary cancer cells.
- 4) It has been known for some time that cross talk exists between ER-mediated events and other signaling pathways (e.g., those regulated by peptide growth factors and their second messenger systems), and the ER itself undergoes phosphorylation/dephosphorylation events that could alter its activity. More recent studies have also identified a number of other nuclear factors, including coactivators, corepressors, and integrator proteins that play important roles in ER-mediated transcriptional events. A key observation is that these factors can alter the magnitude of cellular responses to estrogens and other steroids. Identification of these factors and the mechanisms by which they operate are likely to provide additional indices that can be used in conjunction with ER/PR levels to classify breast tumors and predict the efficacy of current hormonal therapies and to develop new therapeutic targets. A related advance has been the recognition that substantial diversity is found in the location and sequence of EREs in endogenous hormone responsive genes.

and these differences may also increase our understanding of mechanisms by which ER-mediated processes affect breast cancer. Dr. Greene's talk discusses the interaction of several factors with the ER and illustrates that different ligands produce different structural states of the receptor that could interact differentially with other regulatory molecules, such as coactivators and corepressors.

- 5) In addition to the classic ER, now referred to as ER- α , a second receptor termed ER- β has been identified in humans and in animals. The two receptors show different tissue distributions, and, although they have generally similar ligand binding patterns, at least several differences appear to exist. An exciting era of endocrine research will be to define further the properties, distribution, and regulation of these receptors and to identify the biologic responses that they mediate. This new receptor is discussed by Dr. Gustafsson, whose laboratory has been the leader in the identification and characterization of ER- β .

Cellular Components That Distinguish Between Agonist- and Antagonist-Activated Steroid Receptors

Research in Dr. McDonnell's laboratory has been driven in large part by two key issues in estrogen and antiestrogen pharmacology. First is the issue of how to obtain tissue selectivity with estrogens used for hormone replacement therapy. It is clearly established that estrogens diminish vasomotor instability ("hot flashes"), preserve bone mass, and have beneficial effects on cardiovascular health. An emerging view is also based on epidemiologic evidence that they may also benefit cognitive function and delay the onset of Alzheimer's disease. However, it is highly problematic that estrogens used for these desirable purposes produce proliferative effects on the breast and endometrium. It is the fear of breast cancer, in particular, that greatly limits the use of estrogen replacement therapy by many women. The second pharmacologic issue is the use of tamoxifen as an adjuvant treatment of breast cancer. The drug has established efficacy in the treatment of the disease, but tamoxifen treatment generally fails after a period of time, and use for prolonged periods (e.g., 10 years) may actually be less beneficial than use for shorter times (e.g., 5 years). These observations were very difficult to reconcile with a simple mechanism of estrogen action in which "all estrogens are alike" in that they simply activate the receptor, and antiestrogens simply act by "freezing" the ER in an inactive state akin to that of an unliganded receptor protein.

Roughly 5 years ago, a number of studies began appearing that were inconsistent with this simple view of ER activation. One study was a clinical paper published by Love et al. (22) in 1992. These workers examined the effect of tamoxifen on bone mineral density of the lumbar spine in women receiving the drug for the treatment of breast cancer, and their data indicated that tamoxifen increased bone mass. In other words, the drug acts as an estrogen agonist in bone, in contrast to its antiestrogen action in the breast. This study was one of the first well-documented clinical studies of a SERM and clearly indicated that an ER ligand could have opposite effects in different target tissues.

Shortly thereafter, studies in Dr. McDonnell's own laboratory demonstrated that the binding of different ligands caused the ER to assume different conformations (23). In these studies, he used protease digestion to probe subtle differences in ER conformation. When trypsin was incubated with the unliganded ER, the 66-kd molecular weight native receptor was degraded to very

low-molecular-weight fragments. When estradiol was bound to the receptor, however, the receptor assumed a conformation less susceptible to protease digestion because a relatively large receptor fragment (32 kd) remained after prolonged digestion. When tamoxifen was bound to the receptor, the protein was not degraded to very small fragments, indicating that tamoxifen did not simply hold the receptor in an inactive conformation similar to that of the unliganded protein. Rather, tamoxifen binding produced a conformational change that protected a relatively large protein fragment (28 kd) from trypsin digestion. This finding indicated that tamoxifen actually put the ER in a conformation that was distinct from either that produced by the endogenous hormone (estradiol) or that of the unliganded receptor, which was previously presumed to represent its inactive conformation. This study provided physical evidence that different ligands caused the receptor to assume different conformations.

These laboratory investigations suggested a molecular explanation for clinical findings such as those reported by Love et al. (22) in different tissues (i.e., bone versus breast cancer cells). Because tamoxifen and estradiol put the receptor into different conformations, this investigation suggested that the different tissues had ways to functionally "distinguish" structural difference in the receptor (i.e., the conformation of the ER-tamoxifen complex could function as an agonist in bone but not in breast cancer cells). It was known at this time that the ER had a modular structure and that two different regions of the protein could function to activate transcription. One such region, termed transcription-activating function 1 (referred to as either TAF-1 or AF-1 in the literature) was present in the N-terminal region of the receptor, and a second (AF-2) was present in its carboxyl-terminal region. This knowledge raised the possibility that different ligands (e.g., estradiol versus tamoxifen) might put the receptor into conformations in which the two AFs were differentially active. To test this hypothesis, Dr. McDonnell's group performed a series of co-transfection studies with the use of wild-type ERs that contained both AFs and ER mutants in which only one of the AFs was active (24).

A series of such studies indicated that most cultured cells (approximately 90% of those tested) required both AF-1 and AF-2 functions for transcriptional activity when stimulated by estradiol, but the hormone could stimulate transcription in some cells via receptors with only an active AF-1 or an active AF-2 function. Tamoxifen failed to activate transcription in all cases in which both the AF-1 and AF-2 functions were required and in cases in which the AF-2 function alone could mediate estradiol-induced transcription. In these cell types, tamoxifen functioned as a pure estrogen antagonist to block the action of estradiol. In contrast, in those cells in which estradiol could activate transcription from receptors with only a functional AF-1, tamoxifen could act as a partial agonist with substantial estrogen-like activity. These interactions are illustrated schematically in Fig. 2. These studies were also important because they established that tamoxifen could function both as a partial estrogen agonist and as a pure estrogen antagonist via the same receptor system. This finding ruled out the possibility that the antagonist and partial agonist activities of antiestrogens were mediated by different receptor systems.

In the early 1990s, the concept was also emerging that the role of the receptor AF was to serve as "contact" points for the interaction with other cellular proteins involved in transcription control, the so-called coactivators and corepressors. The idea

ESTROGEN RECEPTOR

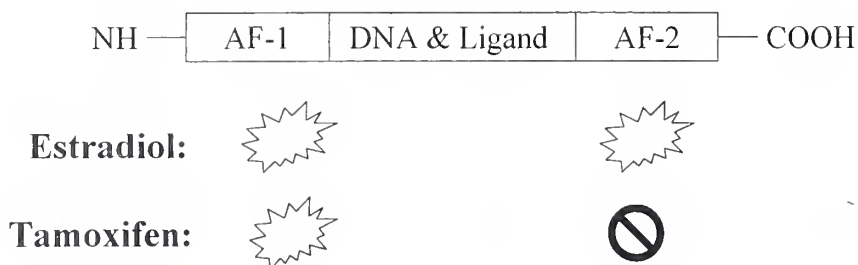


Fig. 2. Agonists and antagonists have differential effects on transcription-activating functions (AF) of the estrogen receptor (ER). The ER has a modular structure involving a N-terminal transcription activation function, termed AF-1, a DNA-binding domain, a ligand-binding domain, and a more C-terminal activation function, termed AF-2. Both estradiol and tamoxifen bind to a similar site in the ligand-binding domain of the receptor. Estradiol is able to activate both AFs. In contrast, tamoxifen prevents activation of AF-2, although the drug can activate the AF-1 function of the receptor.

was that the steroid receptor would bind to EREs in the regulatory regions of target genes and that AF functions (activated by bound hormone) would then recruit these factors, which would alter transcription. This idea raised the possibility that different cells required different AFs in the ER for transcription activation because they contained different complements of coactivators, corepressors, or other regulator proteins. Such differences could be qualitative (i.e., different cells would express different types of proteins) or quantitative (different cells would contain different levels of coactivators/corepressors), or both.

To investigate this possibility, Dr. McDonnell's laboratory performed a series of studies in which they co-transfected one such coactivator (termed GRIP) into cells along with either the wild-type or mutated ER (25). In the cell line used, estradiol produced a full response (100%) of transfected reporter genes with the wild-type receptor, but only a 50% response was produced with receptors in which only AF-1 was active and only 20% in receptors in which only AF-2 was active. However, when vectors expressing GRIP were used to raise cellular levels of this protein, receptors with only AF-1 or AF-2 function produced the same transcriptional response as wild-type receptors. This finding established the principle that coactivators present in some cells are sufficient to enable the ER to activate transcription when only one of its two AFs is activated by ligand binding.

Collectively, studies such as these indicated that tamoxifen could function as a partial agonist if only the AF-1 function of the receptor was required, but it always functioned as an antagonist if the AF-2 function was required, either alone or in combination with the AF-1 function. A related question that Dr. McDonnell also considered was whether all antiestrogens would display this same type of behavior, and he thus began a series of studies to investigate the ability of different antiestrogens to activate AF-1 activity but block AF-2 activity. These studies led to the recent discovery of an antiestrogen, GW-5638, with an activity profile different from that of tamoxifen.

GW-5638 is a triphenylethylene antiestrogen that appears totally devoid of either the AF-1 or AF-2 type of activity (26). This lack of AF activity is not due to poor entry into target cells, because the drug can block the transcriptional effects of estradiol and tamoxifen in cultured cell systems. This drug is thus a pure antiestrogen in the breast, but it retains the ability to maintain bone mass without producing any uterotrophic action in rats. This ability indicates that ER ligands *without* AF-1 or AF-2

activity can function as estrogen agonists in bone. This function suggests that ER-mediated actions in target cells are even more complex than previously recognized and that other factors besides AF-1 and AF-2 functions are likely to be involved in estrogen actions in some cell types.

Role of ER- α in Carcinogenesis With the Use of Transgenic Mouse Models

The estrogen signaling system has long been implicated as a possible factor in the induction and/or promotion of carcinogenesis, especially in the tissues of the female reproductive tract and of the breast. The proliferative effects of the natural ligand, 17 β -estradiol, as well as the synthetic estrogen diethylstilbestrol (DES) in the uterus, vagina, and mammary gland have been well studied. The majority of the cellular effects of estrogens are thought to be mediated by the ER, now known to exist in two types, the well-characterized ER- α and the newly discovered ER- β . Although it has been established that the ER must be present for most estrogen-induced mechanisms, the relationship between the levels of ER and the extent to which a tissue is estrogen responsive is less understood. Furthermore, the influence of varied ER levels in carcinogenesis is even less well known. Efforts to understand further the role of the ER- α in carcinogenesis have led to the generation and characterization of a series of transgenic mouse models that possess altered levels of ER- α expression. The MT-mER mice possess a transgene that results in overexpression of the ER- α protein, whereas the ER α KO mice are homozygous for a targeted disruption of the ER- α gene and, therefore, possess no functional levels of ER- α (27). By using these models, studies have been conducted to elucidate further the role of ER- α in the induction and promotion of hormonally (DES)-induced tumors of the reproductive tract (28) and of oncogene-induced tumors of the mammary gland (29).

In utero exposure to DES, a potent synthetic estrogen, has been linked to a significantly higher risk of a rare form of vaginal cancer, as well as other reproductive abnormalities in humans. The effects of neonatal DES exposure in the female mouse include structural abnormalities in the uterus, oviduct, and bone; uterine tumors; and vaginal adenosis and adenocarcinoma, whereas, in the male, increased incidence of retained testes and hypoplasia of the accessory sex organs have been reported. However, the exact mechanisms by which develop-

mental exposure to DES leads to such abnormalities remain unknown. DES is able to bind ER- α and to mimic the proliferative effects of the natural hormone 17 β -estradiol in the uterus and the vagina. However, DES and its metabolites are also able to directly bind DNA and tubulin, reportedly increasing the incidence of aneuploidy and of nondisjunction in dividing cells. Therefore, it is possible that the developmental and carcinogenic effects of DES may be a direct result of its ER- α -mediated activity, its nonreceptor-mediated genotoxic effects, or both.

The role of ER- α in the induction and promotion of DES-induced tumors was first investigated by using the transgenic MT-mER mice. The uteri of adult MT-mER mice possessed approximately 25% more ER- α than their wild-type littermates (29). It was hypothesized that, because of this abnormal expression of ER- α , the reproductive tract tissues of the MT-mER mice may be more susceptible to tumors after neonatal exposure to DES. Wild-type and MT-mER littermates were treated with DES on days 1–5 at 2 mg/pup per day and then killed at 4, 8, 12, and 18 months of age. At 8 months of age, DES-treated MT-mER mice demonstrated a significantly higher incidence of uterine adenocarcinoma at 73% compared with 46% in the DES-treated wild-type mice (Table 1). These tumors were also preceded at 4 months by a significantly higher incidence of the preneoplastic lesion, atypical hyperplasia, in the MT-mER mice at 26% compared with 0% in the wild-type mice. These data indicate that the level of ER- α present in a tissue may be a determining factor in the progression of estrogen-responsive tumors.

Further studies (30,31) designed to possibly segregate the estrogenic and genotoxic effects of DES have utilized the ER α KO mice, which possess no functional levels of the ER- α protein. Wild-type, heterozygous, and ER α KO littermates were treated as described above in the MT-mER study and also killed at 4, 8, 12, and 18 months. At all time points, uterine weight was significantly reduced in DES-treated wild-type and heterozygous females, whereas no difference was observed in the ER α KO females. Furthermore, the persistent cornification and hyperplasia of the vaginal epithelium as well as the progressive proliferative lesions of the oviduct that are characteristic of neonatal exposure to DES were observed in the wild-type and heterozygous mice but absent in the ER α KO females. At 4 months of age, squamous metaplasia was occasionally observed in the

uteri of DES-treated wild-type females but not in the DES-treated ER α KO mice. In the males, significant atrophy of the seminal vesicle was observed at all time points in both DES-treated wild-type and heterozygous mice, whereas no difference was observed between control and DES-treated ER α KO males. The incidence of tumors, as well as possible altered gene expression in reproductive tract tissues of the different genotype/treatment groups, is currently being assessed. These results thus far indicate that certain developmental effects of DES are, indeed, ER- α mediated.

Finally, the group of Couse and Korach has utilized the ER α KO to study the influence of ER- α in mammary tumors induced by the ectopic expression of the Wnt-1 oncogene. Mice possessing the MMTV-LTR-driven Wnt-1 transgenic construct are known to develop hyperplastic ductal and alveolar epithelium and eventually mammary adenocarcinoma during adulthood (32). Therefore, they have crossed Wnt-1 transgenic mice with the ER α KO mice to generate mice that possess the Wnt-1 transgene on a background of altered ER- α levels (33). The adult female ER α KO mammary gland is completely undeveloped, exhibiting only a rudimentary ductal structure and lacking any terminal end or alveolar buds. However, ectopic expression of the Wnt-1 gene in the ER α KO mammary gland did result in hyperplasia of the existing ductal structure, but it did not lead to further ductal branching or the development of terminal end buds as exhibited by the Wnt-1/wild-type ER- α females. In addition, the average time of tumor onset in the Wnt-1/ER α KO females was much delayed (50% at 48 weeks) compared with the Wnt-1/wild-type ER- α littermates (50% at 24 weeks), even though the serum levels of estradiol in the ER α KO females are approximately 10-fold higher than normal. Postpubertal ovariectomy, as well as pregnancy, had no effect on the growth rate of the mammary tumors in the Wnt-1/wild-type ER- α females. However, prepubertal ovariectomy did result in a delayed average time to tumor onset in the Wnt-1/wild-type ER- α as well as the Wnt-1/ER α KO females compared with that of their respective intact study groups. The results of these studies indicated that Wnt-1-induced mammary tumors can arise in the absence of functional ER- α , as well as ovarian hormones. However, their results have demonstrated that estrogen actions, as mediated by the ER- α , do act to promote the growth of Wnt-1-induced tumors.

Regulation of Cell Cycle and Cell Death in Mammary Cancer

Physiologic levels of estrogens and progestins are well known to promote both onset and malignant progression of breast cancer. A number of investigators in the field believe that an imbalance of mammary epithelial proliferation and death (apoptosis) contributes to tumor formation, genomic instability, and metastasis. They have hypothesized that imbalanced expression of steroid-regulated genes triggers this diverse cascade of processes (34,35). Both estrogenic and progestational steroids are known to regulate expression of genes encoding several polypeptide growth factors, growth factor-binding proteins, and growth factor receptors. In the case of the epidermal growth factor (EGF) family of ligands and receptors (including transforming growth factor- α [TGF- α], amphiregulin, EGF receptor, and c-erbB₂), their pathologic overexpression and functional relevance for breast cancer has received experimental support *in vitro*, *in vivo*, and in ongoing clinical studies. Conversely,

Table 1. Effect of increased ER- α on the progression of DES-induced tumors in the mouse reproductive tract

Observation	Age, mo	No. of affected mice/total No. of mice			
		Control		DES	
		Wild type	MT-mER	Wild type	MT-mER
Squamous metaplasia	4	0/15	0/14	2/19 (11%)	12/19 (63%)*
	8	0/11	0/10	6/24 (25%)	1/26 (4%)
Atypical hyperplasia	4	0/15	0/14	0/19 (0%)	5/19 (26%)*
	8	0/11	0/10	3/24 (12%)	1/26 (4%)
Adenocarcinoma	4	0/15	0/14	0/19 (0%)	0/19 (0%)
	8	0/11	0/10	11/24 (46%)	19/26 (73%)*
	12	0/15	0/15	11/15 (73%)	13/15 (87%)
	18	0/19	0/19	13/14 (93%)	12/13 (92%)

* $P < .05$ as calculated by the Fisher exact probability test, comparing DES-treated MT-mER to DES-treated wild type. Adapted from (28). DES = diethylstilbestrol; ER = estrogen receptor.

growth factors have been shown to regulate expression and function of steroid receptors (34,35). Sex steroids and growth factors appear to exert their principal influences on the cell cycle and cell survival through regulation of cyclin D₁ and Bcl-2/BclX_L, respectively (35–37). The c-myc gene and the bcl-2 gene family have been shown to be important downstream mediators, both of the actions of steroids and of the EGF ligand/receptor family on cellular proliferation and survival; of particular interest, the c-myc gene is amplified in 20%–30% of breast cancer cases and aberrantly expressed in a much higher proportion of cases (38,39). A recent meta-analysis of published clinical pathologic studies (39) has demonstrated that amplification of the c-myc gene is associated with increased lymph node metastases and poorer survival, irrespective of expression of the ER. Myc appears to exert its principal effect on the cell cycle through activation of CDK-2; however, its overexpression sensitizes cells to apoptosis coincident with induction of the proapoptotic p53 and bax genes (38).

As a model system to examine the consequences *in vivo* of deregulated expression of two important, but functionally quite distinct, mediators of the action of estrogen, Dr. Dickson's group has carried out a cross of transgenic Myc- and TGF- α -overexpressing mouse strains. Bitransgenic progeny exhibited a remarkable synergy between the two genes for mammary tumorigenesis, independent of sex, parity, and reproductive hormonal status, indicating that deregulated expression of these two estrogen-inducible genes can entirely supplant an etiologic role for estrogen in malignancy (40). They observed that the mechanism of interaction of Myc and TGF α involved a coordinated stimulation of the cell cycle and suppression of apoptosis (Fig. 3) (41). First, the two gene products interacted such that TGF- α -induced BclX_L allowed cellular survival in the presence of Myc-induced p53 and Bax (36). The EGF receptor-mediated effect on survival appears to depend on signal transduction, both

through the Erk1/Erk2 and the PI3K pathways (42). An independent survival effect is also mediated in these cells by collagen IV acting through a β_1 integrin-PI3K mechanism (43). Second, through their concordant activation of CDK-4 (by induction of cyclin D₁) and CDK-2 (by destruction of the CDK inhibitor p27), the two gene products markedly stimulated aberrant cell cycles and promoted the appearance of multiple chromosomal aberrations (44,45). The results of decreased modulation of p27 by c-myc appear to be sufficiently potent to allow abrogation of the anchorage-independent G₁/S cell cycle checkpoint (46). The appearance of dicentric chromosomes and of a wide array of other chromosomal abnormalities was suggestive of a bridge-break-fusion cycle mechanism at work. The p53 gene was observed not to be mutated in Myc-expressing mammary tumors; it played no obvious role in surveillance of the chromosomal defects observed (47,48). Future studies must further address these mechanisms of cell cycle dysregulation, apoptosis suppression, genomic instability, their relevance to sex steroid action, and their roles in human breast cancer.

ER Structure, Modulators, and Targets in Hormone-Responsive Tissues and Cancers

Dr. Greene emphasized that the ER does not function in a vacuum but that it interacts with many other proteins. For example, it is well established that the ER and other steroid receptors interact with the heat-shock proteins (49) during the initial synthesis of the receptor to ensure its proper folding and trafficking, and, in turn, dissociation of heat-shock proteins seems to be required for the ligand-occupied receptor to activate transcription. He also emphasized that one of the major functions of ligand binding is to change the nature of protein-protein interactions between steroid receptors and other proteins and, conversely, that other proteins can alter the state of the ER independent of ligand binding (e.g., by receptor phosphorylation). In

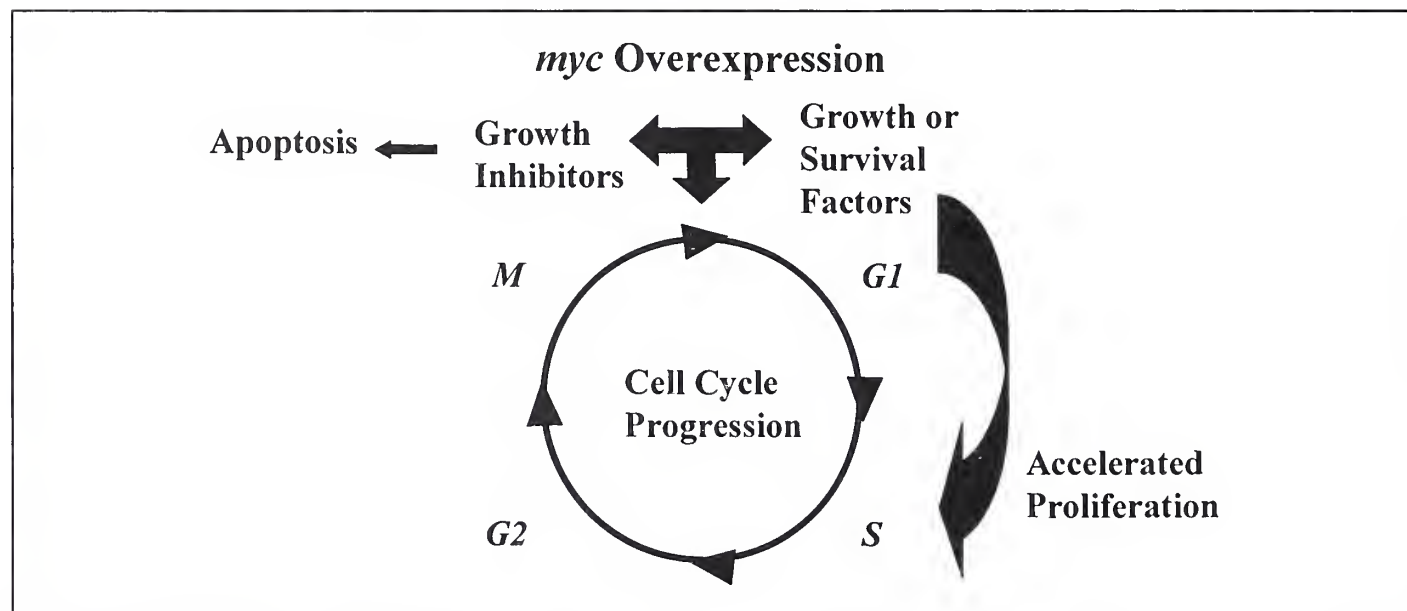


Fig. 3. Model for the dual action of c-myc overexpression in mammary epithelial cells. Deregulated c-myc expression promotes cell-cycle progression through a mechanism that is currently under investigation. The end result of such inappropriate cell-cycle stimulation depends on a number of factors, such as cell genotype and environment. For example, in the presence of certain growth or survival factors (such as activators of the epidermal growth factor receptor or

integrin-mediated adhesion), c-myc expression is proposed to accelerate cell proliferation and promote cell survival. In the absence of such factors or in the presence of certain growth inhibitors (such as transforming growth factor- β), constitutive expression of c-myc is more likely to induce apoptosis [adapted from (38)].

his talk, Dr. Greene presented data from his laboratory on the following three related topics: 1) the identification of gene targets for the ER-ligand complex, 2) the factors that serve to modulate the actions of the ER in target cells, and 3) the structural changes produced in the ER by the binding of different estrogenic and antiestrogenic ligands.

In the first series of studies, Dr. Greene used the technique of RNA differential display to identify transcripts in breast cancer cells that are regulated by estrogens and antiestrogens. This technique identified a number of transcripts with expression altered by ER ligands. Sequence and northern blot analysis of one clone that was decreasingly regulated by estradiol revealed its identity as monocyte chemoattractant protein-1 (MCP-1). The basal expression of MCP-1 is low in MCF-7 breast cancer cells, but it is stimulated by TNF- α , and estradiol blocks induction of the MCP-1 message by the cytokine.

TNF- α is known to regulate MCP-1 expression via the NF- κ B pathway. The MCP-1 gene is known to contain an NF- κ B regulatory element, and reporters containing this element are induced by TNF- α in MCF-7 cells. Despite the fact that reporters containing the MCP-1 promoter do *not* contain any sequences resembling the classic ERE, estradiol blocks induction of such reporters following transfection into breast cancer cells. Extracts from estrogen-treated MCF-7 cells also decrease the binding of NF- κ B to its regulatory element in gel shift studies, and the ER and NF- κ B can be co-immunoprecipitated. Collectively, these studies suggest that the ER blocks TNF- α induction of MCP-1 by directly or indirectly decreasing the binding of NF- κ B to its regulatory site in the 5'-regulatory region of the MCP-1 gene. This mechanism is illustrated schematically in Fig. 4.

TNF- α is known to act via a membrane receptor to stimulate a protein kinase cascade that leads to the phosphorylation of I κ B (Fig. 4). Before phosphorylation, this protein forms a dimeric

complex with NF- κ B in the cytoplasm to prevent its movement to the nucleus. On phosphorylation, the I κ B inhibitor dissociates from the complex and is degraded by an ubiquitin-mediated pathway. This process allows the NF- κ B to translocate to the nucleus, where it binds to NF- κ B sites in target genes and activates their transcription. The ER appears to decrease transcription by preventing the binding of NF- κ B to its regulatory site in the 5'-flanking region of the gene, most likely by a direct interaction of the two proteins, as illustrated in Fig. 4. This finding emphasizes that estrogens and antiestrogens can regulate expression of target genes that do not contain hormone response elements, and such regulation is thus likely to occur via protein-protein interactions. Another recent similar example of regulation by protein-protein interactions occurs via binding of the ER to AP-1 components (50,51).

A second series of studies was aimed at identifying cellular factors that modulate the activity of the ER. To identify such factors, Dr. Greene and his colleagues utilized the ligand-binding domain B (52) of the ER to "capture" proteins from breast cancer cells that interact with the receptor (53). In this approach, a fusion protein between glutathione S-transferase and the ligand-binding domain of the ER is used as an affinity matrix to bind proteins in cell extracts that bind to this domain of the receptor in the presence or absence of estrogens and/or antiestrogens. At present, this approach has already identified at least five to six proteins from MCF-7 cell extracts that bind to the ER.

One such "modulator" protein, which has been identified, is a kinase that binds to the ER in the presence of estrogenic ligands and dissociates from the receptor when it is liganded with antiestrogens, such as hydroxy-tamoxifen. This action enabled the kinase to be purified by first binding proteins in cell extracts to glutathione S-transferase-ER in the presence of estradiol, followed by washing to remove unwanted proteins, and then eluting in the presence of antiestrogens. Similar purification

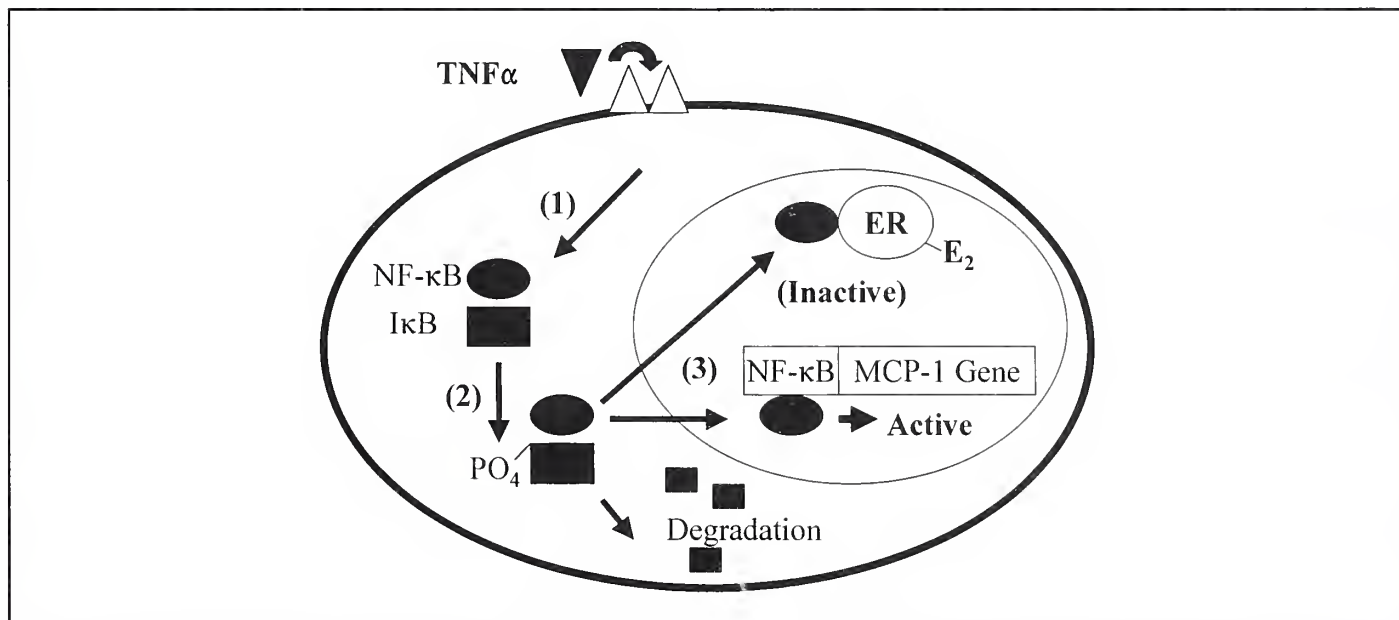


Fig. 4. Proposed model for regulation of monocyte chemoattractant protein-1 (MCP-1) in human breast cancer cells. Tumor necrosis factor- α binds to a membrane receptor and initiates a kinase cascade (step 1) that phosphorylates the I κ B protein shown as a filled rectangle (step 2). Before phosphorylation, I κ B forms a dimer with the transcription factor NF- κ B (filled oval) in the cytoplasm to prevent its entry to the nucleus. Following phosphorylation, I κ B is degraded,

and the free NF- κ B can translocate to the nucleus (step 3). In the absence of estrogens, NF- κ B is "active" as a transcription factor and drives transcription of the MCP-1 gene via a regulatory element in the 5'-flanking region of the gene. When occupied by estradiol, the estrogen receptor (open oval) forms a nuclear complex with NF- κ B so that it is inactive as a transcription factor.

steps were performed by displacing the ER-bound kinase with a peptide with sequences similar to the motifs in coactivators that bind to steroid receptors. This kinase phosphorylates the ER on a serine residue, although the functional consequences of phosphorylation at this site are unknown at present. This series of experiments also emphasizes that protein-protein interactions are increasingly being recognized as playing potential roles in estrogen action.

In a third major series of studies, Dr. Greene and a number of colleagues examined the effects of ligand binding on the structure of the ER. In this work, they solved the crystal structures of the human ER ligand-binding domain (amino acids 301–553) complexed with either estradiol or the mixed antagonist raloxifene (54). This study provided definitive evidence that different ligands produce distinct structural alterations in the receptor. The crystal structures revealed that both ligands bind to the same site within the core of the ligand-binding domain of the receptor, but the two ligands induce major conformational differences in the positioning of the most c-terminal a helix in the receptor (helix-12). This evidence is of major significance because helix-12 is located in the transactivation domain of the ER and appears to be a major site for contact with coactivators and corepressors. Hence, the different structures observed following binding of the two ligands are expected to interact quite differently with these accessory proteins, which drive the transcriptional responses to steroid hormones.

Structure and Function of ER- β

A novel form of the ER, termed ER- β , was originally cloned from the rat prostate (55) and has also been identified in the mouse (56) and in humans (57). The rat ER- β complementary DNA encodes a protein of 485 amino acids with a predicted molecular weight of 54 200 that is highly homologous to the ER- α , particularly in the DNA-binding domain (97% amino acid homology) and the c-terminal ligand-binding domain (59% homology). The amino acid homologies between the three ER- β s identified to date and the human ER- α are illustrated in Table 2 (58), and it is clearly seen that there is nearly perfect homology in the DNA-binding domains of the α and β receptors, substantial homology in the ligand-binding domains, but far less homology in the N-terminal regions. The genes for the two receptors are on separate chromosomes in the human—ER- β on chromosome 14 and ER- α on chromosome 6—removing all doubts that the two receptors are totally distinct species. The major difference in the structures of the two receptors is in the N-terminal region, which is considerably shortened in the β -receptor (58). In addition, another form of the ER- β is present in the rat that contains an in-frame insertion of 54 nucleotides

coding for an insertion of 18 amino acids with the ligand-binding domain. A major difference in the genes for the two ERs is that the α -gene is much larger (approximately threefold) than the β -gene, which has led to the speculation that the latter might be preferentially expressed at certain times in development when shorter genes are more rapidly transcribed, but this speculation remains to be established.

Despite considerable differences in sequence in the ligand-binding domain, both ER- α and ER- β bind the endogenous hormone 17 β -estradiol with about the same affinity. Of interest, however, the β -receptor seems to bind some androgens (e.g., 5-androstenedione) with reasonable affinity, leading to the speculation that this receptor might be activated by androgenic steroids in some situations. In contrast to binding of estradiol, the two receptors show differences in the binding of phytoestrogens, such as genistein and coumestrol, with ER- β having substantially better affinity for these compounds than its α counterpart (59).

It is also clear that both receptors can stimulate transcription from the consensus ERE and that phytoestrogens, as well as estradiol, can stimulate the transcriptional activity of both receptors (59). In addition, ER- α and ER- β are able to form heterodimers that have transcriptional activity when assayed with the traditional ERE. It now appears that both receptors may also stimulate transcription by other non-ERE mechanisms, such as protein-protein interactions with AP-1 components. Of interest, classic ER- α agonists, such as estradiol and DES, function as antagonists in situations in which ER- β stimulates transcription via such AP-1-dependent mechanisms, whereas classic antagonists, such as tamoxifen, act as agonists (51).

In the prostate, ER- β appears to be under androgenic regulation, because its levels decrease with castration and can be restored by testosterone administration. Its expression in the adult prostate is highest in the epithelium and very low in the stroma. This pattern is developmentally regulated, however, because ER- β is present at high levels in both the epithelial and mesenchymal layers of the tissue at birth but is then lost from the stroma in the adult. ER- β does not appear to be regulated by ER- α , because levels of the β receptor are similar in wild-type and ER α KO mice. Although estrogens do not seem to directly regulate ER- β expression, neonatal estrogen treatment appears to decrease the expression of this receptor in certain regions of the adult prostate (60).

Dr. Gustafsson and his colleagues have also investigated the expression of ER- β in models of vascular injury because estrogens appear to offer protection against atherosclerotic disease. By using a model of aortic lesions in mice, it is established that estrogens promote healing and that this effect occurs equally in ER α KO mice and wild-type animals (61). This finding suggests that ER- β may have an important role in the vascular response to estrogens. Of interest, ER- β expression (but not ER- α) is dramatically increased in both the endothelial cells and smooth muscle cells following vascular injury, again suggesting a potential protective role for estrogens acting via ER- β .

In the female reproductive system, ER- β may play a prominent role in the ovary. During follicular development, the granulosa cells express high amounts of this receptor, and its level seems to associate with mitotic activity, whereas little receptor is seen in the thecal cells. During the second half of the cycle, ER- β levels then decline. The β -receptor is also widespread through the urogenital tract, leading to speculation that it may

Table 2. Homologies among various ERs, given as percentage of amino acid identity to the human ER- β *

Receptor	Domain					Overall
	NH-term	DBD	Hinge	LBD	F	
Human β	100.0	100.0	100.0	100.0	100.0	100.0
Human α	17.5	97.0	30.0	59.0	17.9	47.0
Mouse β	80.6	98.5	84.4	91.9	78.6	88.0
Rat β	79.6	98.5	85.6	93.4	78.6	89.0

*ER = estrogen receptor; NH-term = amino terminal; DBD = DNA-binding domain; hinge = Hinge region; LBD = ligand-binding domain; F = F domain.

mediate estrogen action on tissues such as the bladder. In this regard, there are many anecdotal reports that estrogen replacement has beneficial effects on urogenital atrophy and micturition in postmenopausal women even though the bladder does not appear to contain significant levels of ER- α , thus suggesting that the newly discovered ER- β may mediate these actions. Another major site of ER- β expression in female animals is the mammary epithelium of pregnant animals. This finding is particularly significant because these cells have previously been reported *not* to express ER- α , and it was thus thought that estrogen effects on these epithelial cells were mediated by stromal ERs. This recent finding now suggests that ER- β may directly mediate hormonal actions on the mammary epithelium.

Because of increasing interest in the possible actions of estrogens on cognitive function and Alzheimer's disease, Dr. Gustafsson and his colleagues have compared the expression patterns of ER- α and ER- β in the central nervous system of developing and mature rodents. Expression of both receptors is widespread in the central nervous system, but differences are seen in the relative expression in different brain regions (62), suggesting that the two receptors may mediate different functions in the brain. One speculation is that the α -receptor may play a more prominent role in reproductive behaviors and the β -receptor might play an important role in certain aspects of cognitive function, but these roles remain to be established.

Other sites in which the ER- β shows substantial levels of expression include the bone, kidney, lung, adrenal cortex, intestinal mucosa, lymph nodes, testis, sperm, thymus, spleen, and peripheral leukocytes. This expression raises the possibility that ER- β -selective agonists and antagonists might be able to produce selective effects in such tissues. These selective effects might offer some distinct advantages (e.g., for hormone replacement therapy, when one wishes to minimize the hyperproliferative actions of estrogens on the endometrium and breast). The possibility for producing selective estrogenic actions via this newly discovered receptor has thus prompted the search for such receptor selective agents.

SUMMARY AND POTENTIAL IMPLICATIONS FOR HORMONAL CARCINOGENESIS

A number of key points emerged from this session that are likely to have particular relevance for understanding the transcriptional actions of estrogens and antiestrogens in breast, prostate, and other cancers.

- 1) Studies with transgenic animals overexpressing the ER- α have clearly shown that the level of expression of this protein can affect the rate of progression of several cancers. In addition, it is now clear that the ER interacts with a large number of other proteins to regulate transcription. These proteins include the so-called coactivators and corepressors as well as a variety of other regulatory proteins [for recent reviews, see (63–65)]. Thus, in addition to the levels of the classic ER- α and the newly discovered ER- β , the levels and activities of these proteins may affect the etiology of hormone-dependent cancers, their growth responses to estrogenic substances, and their response to hormonal therapies.
- 2) It is now clear that different estrogens and antiestrogens can have differential effects on the multiple activation functions of ERs and that these activation functions provide the surfaces that interact with coactivators and corepressors to regu-

late target gene expression. This finding has radically changed our thinking about the pharmacology of estrogens, and it now appears theoretically feasible to design highly selective estrogens with minimal growth-promoting effects on breast and other tumors (66,67). Conversely, this finding raises the possibility that certain estrogens might play a greater role in breast and prostate cancers than in others.

- 3) At the cellular level, it is now clear that we must consider hormone and antihormone effects on cell death, as well as cell proliferation. We must also understand how estrogens affect the interaction of cells with their environment (e.g., substratum, vascular system, etc.) as well as mechanisms by which estrogens regulate internal production of factors that regulate cell function at intracellular sites and understand the basis for the genomic instability commonly seen in cancer cells.
- 4) One area briefly mentioned by Dr. Greene was the possibility that antiestrogens may play a more "active" role in the treatment of breast cancer than the simple competitive blockade of estrogen actions at the receptor site. Thus, several reports are available that antiestrogens, acting through the ER or other mechanisms, may induce the synthesis of factors normally suppressed by estrogens, or at least not expressed in the absence of antiestrogens. One area of great potential significance when considering estrogens as endogenous carcinogens are the reports that antiestrogens may induce expression of quinone reductase (68,69). This action would certainly be expected to decrease effects of reactive estrogenic metabolites, such as catechol estrogen quinones, or other genotoxic chemical species generated by redox cycling.

Finally, it should be emphasized that, although there is increasing interest in understanding the role of estrogens as endogenous carcinogens (Chapters 3–5) that may have actions independent of the classic ER, it is clear that ER-mediated processes play significant roles in normal and cancer cells. An increased knowledge of both types of processes is thus certain to enhance our understanding of the causes, treatments, and mechanisms to prevent many of the most prevalent human cancers.

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NOTE

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Chapter 9: Factors Critical to the Design and Execution of Epidemiologic Studies and Description of an Innovative Technology to Follow the Progression From Normal to Cancer Tissue

Montserrat Garcia-Closas, Susan E. Hankinson, Shuk-mei Ho, Donald C. Malins, Nayak L. Polissar, Stefan N. Schaefer, Yingzhong Su, Mark A. Vinson

The results obtained from experimental studies of estrogen carcinogenesis need validation in epidemiologic studies. Such studies present additional challenges, however, because variations in human populations are much greater than those in experimental systems and in animal models. Because epidemiologic studies are often used to evaluate modest differences in risk factors, it is essential to minimize sources of errors and to maximize sensitivity, reproducibility, and specificity. In the first part of this chapter, critical factors in designing and executing epidemiologic studies, as well as the influence of sample collection, processing, and storage on data reliability, are discussed. One of the most important requirements is attaining sufficient statistical power to assess small genetic effects and to evaluate interactions between genetic and environmental factors. The second part of this chapter describes innovative technology, namely, Fourier transform-infrared (FT-IR) spectra of DNA that reveal major structural differences at various stages of the progression from normal to cancer tissue. The structural differences become evident from wavenumber-by-wavenumber statistical comparisons of the mean FT-IR spectra of DNA from normal to cancer tissues. This analysis has allowed distinguishing benign tissues from cancer and metastatic tissues in human breast, prostate, and ovarian cancers. This analysis, which requires less than 1 μ g of DNA, is predicted to be used for detecting early cancer-related changes at the level of DNA, rather than at the cellular level. [J Natl Cancer Inst Monogr 2000;27:147–56]

In the study of estrogen carcinogenesis, it has become apparent that results obtained from experimental studies need validation in epidemiology/population studies. However, because variations in a human population are much greater than those existing in experimental systems and in animal models, population studies present additional challenges. In addition, because epidemiologic studies are frequently used to evaluate modest differences in risk factors and, therefore, in their design, it is essential to minimize sources of errors and technical variations and to choose methods with maximum sensitivity, reproducibility, and specificity. In these regards, this chapter focuses on two important topics: One deals with technical issues in study design and in statistical power requirements, and the other focuses on the development of a new technology to measure a surrogate cancer risk marker.

Several methodologic challenges and technical hurdles in designing and executing epidemiologic/population studies are discussed in this chapter. Important issues that are discussed include reproducibility of laboratory assays, limitations imposed

by the small amount of plasma/serum collected, and the validity of using a single sample per subject. The chapter also discusses in detail the influences of sample collection, processing, and storage methods on data reliability. Finally, the importance of attaining adequate statistical power by reaching the required sample sizes is highlighted.

Several important lessons are enumerated. 1) Collection protocols need to minimize variations in factors that are not of etiologic interest by standardizing case and control subjects on these factors. 2) Sample collection, processing, and storage procedures must be subjected to stringent scrutiny to ensure that variations in these steps will not mask the modest differences expected to exist in the risk factors of interest. 3) Study design must take into consideration the limitations linked to within-person variation over time as well as the single sample per subject collection method and, therefore, whenever possible, repeated sampling should be considered. 4) Comparison of data collected from different laboratories may be difficult because large variations exist in different study populations and in laboratory methods. Introduction of a standardization or validation program should be considered for multisite analyses. 5) To attain statistical power in case-control studies, larger sample sizes are needed for studies that are assessing small genetic effects. Furthermore, if the goal of the study is to evaluate interaction among factors, sample sizes need to be increased accordingly.

The second half of this chapter focuses on breakthrough technology referred to as the Fourier transform-infrared/statistics model, which has been successfully adapted for analyses of DNA changes in cancer and precancerous tissues (1–6). Infrared spectra generated by applying infrared beams to sample DNA produced a large number of spectra. Fourier transform spectral data analyses, coupled with statistical comparisons, yield a few

Affiliation of authors: M. Garcia-Closas, Environmental Epidemiology Branch, National Cancer Institute, Bethesda, MD; S. E. Hankinson, Department of Epidemiology, Harvard School of Public Health and Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA; S. Ho, University of Massachusetts Medical School, Department of Surgery, Division of Urology, University Campus, Worcester; D. C. Malins, Molecular Epidemiology Program, Pacific Northwest Research Institute, Seattle, WA; N. L. Polissar, The Mountain-Whisper Light Statistical Consulting, Seattle, and Department of Biostatistics, University of Washington, Seattle; S. N. Schaefer, Y. Su, M. A. Vinson, Molecular Epidemiology Program, Pacific Northwest Research Institute, Seattle.

Correspondence to: Shuk-mei Ho, Ph.D., University of Massachusetts Medical School, Department of Surgery, Division of Urology, Office of Urologic and Translational Research, 55 Lake Ave., No., Worcester, MA (e-mail: Shuk-Mei.Ho@UMASSMED.EDU).

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principal components that were shown to be sufficient for discriminating DNA alterations in precancerous and cancer DNA samples. The technology has recently been developed further to produce cancer probability-risk score models for breast and prostate cancers. With the use of a model developed for breast cancer, normal tissue, primary tumors, and metastasizing tumors were correctly discriminated at more than 80% probability. The method also has the added advantage of requiring only a small amount of DNA (<1.0 μ g) and, therefore, is potentially suitable for analyses of needle biopsy samples. Should the technology live up to its promise, it will provide a highly sensitive and reliable diagnostic and risk-assessment method for clinical and population studies.

STUDY DESIGN CONSIDERATIONS IN THE ASSESSMENT OF CANCER RISK IN RELATION TO GENETIC POLYMORPHISMS

Polymorphisms in genes coding for enzymes or receptors involved in the metabolism and intracellular transport of estrogens could influence the risk of developing breast cancer (7). The case-control design is the most commonly used to evaluate associations between genetic polymorphisms and the risk of common diseases in the population, as well as interactions between genetic and environmental risk factors (8). In this type of study, the odds ratio is used to measure the association between a particular genotype and the risk of disease. The number of case and control subjects that would need to be included in such studies to have an 80% power (two-sided test with 5% Type I error) to detect a genotype effect, as a function of the prevalence of the genotype and the magnitude of the effect, is illustrated in Fig. 1. According to Fig. 1, studies including 200 case patients and 200 control subjects or fewer will be able to detect moderate to large genetic effects (odds ratio [OR] ≥ 2.0) for a wide range of genotype prevalences. However, a minimum of 400 case patients and 400 control subjects will be needed to detect small genetic effects (OR = 1.5).

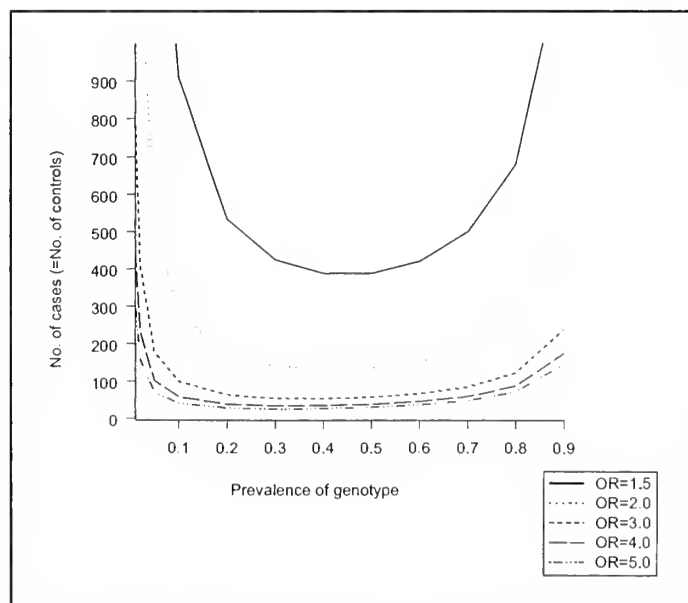


Fig. 1. Number of case patients required to have an 80% power to detect a genotype effect with the use of a two-sided test with 5% Type I error, as a function of genotype prevalence and magnitude of effect.

It is likely that a gene coding for a particular metabolizing enzyme confers disease susceptibility in combination with genes coding for other enzymes involved in the same metabolic pathway or in combination with other determinants of substrate levels. Therefore, studies should be designed to be able to evaluate potential gene-gene and gene-environment interactions. The assessment of interactions requires large sample sizes to attain adequate statistical power, especially when the factors under study are either very rare or very common or when the magnitude of the interaction is modest (9-12). Estrogen-related risk factors, such as reproductive characteristics or body size, have small to moderate effects on breast cancer risk, and susceptibility genotypes, such as those for metabolizing enzymes, are also likely to have small to moderate effects. Therefore, we expect to observe modest interactions, unless the genetic or environmental factors are very rare, and, in both situations, large samples are needed. The sample size requirements to have an 80% power to detect an example of a twofold multiplicative interaction (ratio of stratum-specific ORs of 2.0), as a function of the prevalence of the environmental and genetic factors, is presented in Fig. 2. In this example, it is assumed that the genotype effect in the absence of the exposure and the exposure effect in the absence of the genotype are both 2.0 and that the genetic and environmental factors occur independently in the population. This figure indicates that a minimum of about 550 case patients and 550 control subjects will be needed to study this type of interaction (genotype and exposure prevalence of 30%-50%), and a much larger sample size will be required to study less common or more common genetic or environmental factors, especially if prevalences are lower than 20%. Similarly, the sample size will increase as the genotype or exposure prevalence becomes larger than 30%-50%.

The sample size to study multiplicative interactions is mainly determined by the susceptibility genotype and exposure prevalence and by the magnitude of the interaction (11). The magnitude of the effect of the genotype in the absence of the exposure and the effect of the exposure in the absence of the genotype

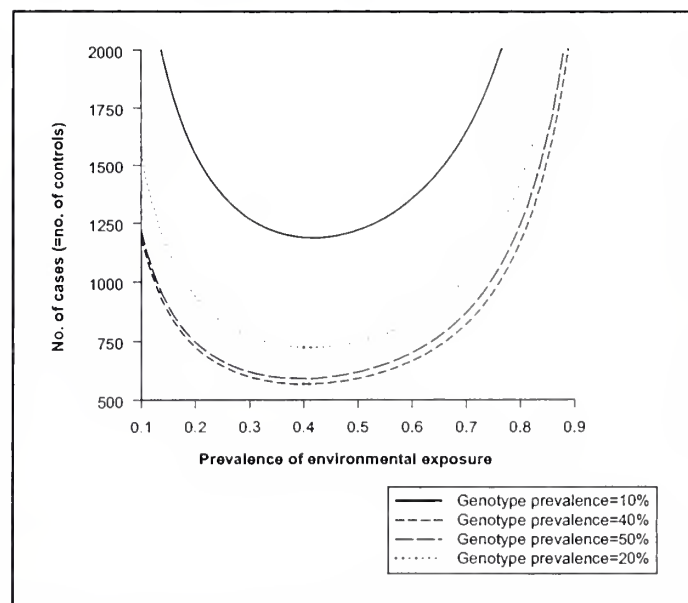


Fig. 2. Number of case patients required to have an 80% power to detect an example of a twofold gene-environment interaction with the use of a two-sided test with 5% Type I error, as a function of genotype and exposure prevalence.

affect the sample size to a lesser extent. Thus, the sample sizes illustrated in Fig. 2 will be similar for other examples of twofold gene–environment interactions in which the genotype or exposure effects are smaller than 2.0. Moreover, the sample size needed to study a 1.5-fold interaction, rather than a twofold interaction, will be two to three times higher.

Accurate information is essential for the study of environmental exposures and their potential interactions with genetic polymorphisms (13,14). Misclassification of exposure, either differential or nondifferential with respect to disease status, will tend to underestimate a multiplicative interaction effect, provided that exposure misclassification is independent of the genotype and that the exposure status is independent of the genotype in the population (15). As a consequence, the sample size required to detect the attenuated interaction with adequate statistical power will be increased.

The impact of misclassification on estimation and sample size is highly dependent on the exposure prevalence (13,14). For instance, the use of an instrument with low sensitivity (proportion of exposed subjects correctly classified) can have a very strong impact when the exposure frequency is high, but it may not be so deleterious when the frequency is low. It should also be taken into account that even small errors in the genotype determination can have substantial impact on the sample size, especially when environmental exposure is also measured with error. The impact of exposure and genotype misclassification on sample size, when the prevalence of both factors is 50%, is illustrated in Table 1. The sample sizes correspond to the same example of gene–environment interaction as in Fig. 2. In the absence of genotype misclassification, an exposure sensitivity of 80% increases the sample size about 1.7 times (from 590 to 1600 subjects); in the absence of exposure misclassification, a genotype sensitivity of 95% increases the sample size 0.5 times (from 590 to 900 subjects). However, in the presence of both genotype and exposure misclassification, the sample size will be further increased to 2045 case patients and 2045 control subjects.

Alternative study designs can be more efficient to study interactions under certain circumstances and can address potential biases common in case–control studies (10,16,17). For instance, the case-only design can be very efficient in detecting gene–gene and gene–environment interactions, provided that the genes and the environmental exposure are independent in the population; the case–parental control design addresses the problem of confounding of gene effects by ethnicity or population stratification; and cohort studies can address the problem of disease bias because biologic samples and questionnaire data are collected prior to the onset of disease. Mixed study designs or different sampling strategies, such as oversampling women with

a positive family history of breast cancer to increase the frequency of potential susceptibility genotypes, may also help to address potential biases or increase the efficiency to study particular hypotheses.

SEX STEROID HORMONES: TECHNICAL HURDLES IN POPULATION STUDIES

Challenges in Epidemiologic Studies

The epidemiologic study of steroid hormones in relation to disease risk poses several methodologic challenges. Frequently, we wish to detect only modest (but etiologically important) differences in hormone levels between study subjects. To optimize the chance of detecting these differences, sources of error and technical variation in our results must be minimized. Protocols for the collection, processing, and storage of study urine or blood samples must be evaluated and optimized. Laboratory assay procedures must be reproducible, require a small sample volume, and, preferably, be consistent across studies. In addition, the ability of a single hormone measurement (as is available in most population studies) to reflect long-term hormone levels must be evaluated. Each of these issues is discussed below.

Blood or Urine Collection, Processing, and Storage

Sample Collection

It is important to establish a specific collection protocol that minimizes the sources of variation in hormone levels that are not of etiologic interest. These sources of variation include fasting status, time of day the sample is collected, and, for premenopausal women, phase of the menstrual cycle. Not standardizing case and control subjects on these factors (in either the design or analysis phase of a study) could substantially attenuate hormone/disease associations or, if the distribution of these factors varied by chance between case and control groups, an association may be detected that does not in truth exist. For example, several adrenal androgens [e.g., dehydroepiandrosterone (DHEA)] and prolactin have substantial diurnal variation (18).

Collecting all blood samples at a single time in the day, matching control subjects to case patients on the time of sample collection, or controlling for time of day of collection in the statistical analysis will remove any noise associated with the circadian variation in hormone levels.

For premenopausal women, the effect of the menstrual cycle on hormone levels is important to consider. A number of hormones, particularly estrogens, fluctuate substantially over the menstrual cycle (18). Thus, similarly to what was described above, to allow a valid comparison between case and control subjects, it is necessary to either collect all samples at approximately the same time in the cycle, match on cycle day, or carefully control for cycle day in the analysis. In general, timing the luteal sample from the first day of the next menstrual cycle is more accurate than counting from day 1 of the current cycle, as the luteal phase is more consistent in length than the follicular phase (19). Accurate matching in the luteal phase requires knowing when the next menstrual cycle began (i.e., the cycle after sample collection). This requires recontacting study participants (or having the participants recontact study staff by mail, for example), thus adding an additional challenge to epidemiologic studies of premenopausal hormone levels. It is in part due to the complexity of collecting well-timed samples from premeno-

Table 1. Impact of exposure and genotype misclassification on sample size to detect a twofold interaction*

Scenario	Exposure sensitivity, %	Genotype sensitivity, %	No. of subjects
No misclassification	100	100	590
Exposure misclassification	80	100	1600
Genotype misclassification	100	95	900
Exposure and genotype misclassification	80	95	2045

*Interaction model described in text; prevalence of both genotype and exposure = 50%; genotype and exposure specificity = 100%.

pausal women that few studies of premenopausal endogenous hormones and cancer risk have been conducted.

Sample Processing

Ideally, biologic samples would be processed and either analyzed or frozen immediately after sample collection. This action will minimize any deterioration in hormone levels over time. However, in some large population studies, particularly those with a geographically dispersed population, immediate processing and storage are not feasible. The effect of delayed processing and storage on any parameter of interest must be evaluated before study implementation.

Prior to a large blood collection effort in the Nurses' Health Study, the effect of a delay in blood processing on steroid hormone levels was evaluated (Table 2). The stability of endogenous hormones in plasma prepared from whole blood that had been stored for 24 or 48 hours in a sealed Styrofoam mailer cooled with a frozen gel pack was evaluated relative to samples that were immediately processed and frozen (20). Overall, the delay in sample processing resulted in little change in hormone levels. Estradiol, percentage of free estradiol, androstenedione, and prolactin all changed by less than 5% per day. The hormone with the greatest percentage of change per day was testosterone (9.5% per day). However, even with this degree of change, the true between-person variation substantially outweighed the error introduced by the delay in processing and the laboratory analysis, as evidenced by the high intraclass correlation coefficient [$ICC = 0.86$; i.e., $\text{between-person variation}/(\text{between-person variation} + \text{within-person variation})$]. More recently, these blood collection methods have also been evaluated for their effect on plasma insulin-like growth factor-I (IGF-I) and insulin-like growth factor-binding protein 3 (IGFBP-3) levels (21). IGF-I and IGFBP-3 levels in samples that were processed and serum frozen immediately after venipuncture (the standard processing methods) were compared with samples that were stored in heparinized whole blood for 24–36 hours before processing (mimicking blood collection conditions used in certain studies). The mean IGF-I and IGFBP-3 values were almost identical, and the intraclass correlations between results of the two collection methods were 0.98 for IGF-I and 0.96 for IGFBP-3—again showing that the collection methods did not adversely affect sample integrity.

Sample Storage

Freezer alarm and back-up systems must be in place to prevent thawing or warming of study samples. Twenty-four-hour

alarms should be in place, and manual checks of the freezer temperature should be conducted periodically. For added security, each individual's sample should be split between freezers, so that, in the event that a freezer thaws, only part of a sample from any one participant will be lost. To maintain the ability to identify stored samples, cryotubes should be labeled before freezing, using labels with adhesive specifically designed for low temperatures.

Several different freezing options are available: storage in mechanical freezers at either 20°C or at -70°C, or in the vapor phase (temperature range, -130°C to -196°C) or the liquid phase (constant at -196°C) of liquid nitrogen freezers. Some concern regarding the suitability of upright front-loading mechanical freezers for long-term sample storage was raised in a study conducted by Su et al. (22). They evaluated temperature variations in upright mechanical freezers and found that, for freezers set at -80°C, the internal temperature ranged from -90°C to -43.5°C, with the warmer regions being the upper and front sections of the freezers. We are unaware of similar evaluations in chest freezers.

Several studies have used frozen specimens with little if any sign of degradation in the hormone level. Mean levels of plasma testosterone, estradiol, androstenedione, and percentage of free estradiol in samples stored at -70°C for either 6 or 8 years were not significantly different, although estrone levels were slightly higher in samples stored for only 6 years (23). Plasma estradiol, sex hormone-binding globulin (SHBG)-bound estradiol, free estradiol, and prolactin levels remained stable after archiving at -70°C for 6 months to 6 years (24,25). In other study (26), both estradiol and prolactin were observed to be stable at -70°C for 3 years; although testosterone levels varied modestly over the 3 years, the rank correlation remained high (approximate $r = .9$). In contrast to other steroids, plasma progesterone levels were reported to decrease by 40% over a 3-year period, although, again, the rank correlation over time was high ($r = .98$). However, in a second study (27) in which plasma was stored at -20°C, progesterone levels were reported to increase 2.8% per year of storage. Thus, the stability of plasma or serum progesterone levels over time is uncertain, and additional evaluations are needed. Stability of samples in -70°C or colder over a period of more than 8 years has not yet been evaluated. Although DHEA sulfate has been reported stable when stored at -20°C for 10–15 years (28), the percentage of free (versus bound) estradiol (29) and testosterone (30) has been reported to increase significantly with storage at this temperature; thus, freezing at -70°C or colder is preferred.

Although the above studies suggest that hormone levels tend

Table 2. Plasma hormone levels from postmenopausal women according to delay in processing specimens after phlebotomy

Hormone	Mean hormone levels according to delay in processing after phlebotomy			% change per day*	CV _b /CV _w †
	0 h	24 h	48 h		
Androstenedione, ng/L	516	493	482	-3.4 (-6.9, 0.2)	3.0
Estradiol, ng/L	19	19	20	3.5 (-1.7, 8.7)	1.5
% free estradiol‡	1.8	1.9	1.8	-1.3 (-4.7, 2.1)	4.0
Prolactin, mg/L	8.8	8.9	8.8	-0.4 (-1.9, 2.7)	9.2
Sex hormone-binding globulin, nmol/L	31.3	30.1	32.1	1.3 (-2.4, 5.0)	4.4
Testosterone, ng/L	221	242	262	9.5 (1.7, 17.3)	3.5

*Numbers in parentheses = 95% confidence intervals associated with the percent change per day.

†Ratio of the between-person (CV_b) and within-person (CV_w) coefficients of variation.

‡n = 5; all others, n = 9 subjects.

to be stable for relatively long periods if stored at -70°C or colder, it remains advisable to match case patients and control subjects on length of sample storage to minimize the effect of any modest changes on case/control comparisons. In addition, we are not aware of data that address the stability of hormone levels with repeated freezing and thawing of the biologic material; thus, it is recommended that freeze/thaw cycles be minimized.

Validity of Using a Single Sample per Subject

Although average long-term hormone levels are often of primary interest in epidemiologic studies of hormones and cancer, for both economic and logistical reasons, frequently only one blood sample is collected per study subject. The degree to which this one sample can represent an individual's long-term levels depends on the degree of within-person variation (relative to the between-person variation) over time. The more representative a single sample is of long-term levels, the greater the chance of detecting true differences between study subjects. This issue has been evaluated in several studies.

Correlations for plasma estrogens over approximately a 2-year period in postmenopausal women ranged from 0.36 (31) to 0.94 for the percentage of bioavailable estradiol (Table 3) (25). How well a single postmenopausal hormone measure reflects levels over a 3-year period was evaluated and an ICC of 0.66–0.92 was found for the sex steroid hormones (32). Prolactin had a somewhat lower ICC of 0.53. For IGF-I, among 24 adults who had two blood samples drawn on average 6 weeks apart, the ICC was 0.94 ($P = .001$) (33).

In a study of 26 premenopausal women in which two luteal phase samples were collected about 1.5 years apart, the correlation for the repeated samples was 0.70 for androstenedione and 0.73 for testosterone (Table 3) (34). In samples from 71 premenopausal women that were collected randomly throughout the menstrual cycle over a 1- to 2-year period, the ICC was 0.72

(95% confidence interval [CI] = 0.57–0.83) for percentage of free estradiol and 0.83 (95% CI = 0.73–0.90) for SHBG-bound estradiol ("nonbioavailable" estradiol) (25). In the same study (24), the ICC for prolactin was 0.48 (95% CI = 0.31–0.63). More recently, repeated luteal phase samples were collected 1 year apart from 60 premenopausal women; the ICCs were 0.85 for DHEA sulfate and 0.60 for total testosterone (35). In a study of urinary estrogen metabolite levels, the ICC for the ratio of urinary 2-hydroxyestrone to 16- α -hydroxyestrone over a 6-month period was 0.67 (36).

In the study by Muti et al. (35), the ICC for estradiol in the luteal phase was reported to be just 0.06, suggesting extremely poor reproducibility over time. However, this result may be related to the investigators' inability to exclude women with anovulatory cycles or to pinpoint when in the menstrual cycle the sample was collected. (In this study, the samples were collected on days 20–24, counting forward from day 1 of the cycle, and the date of start of the next menstrual cycle was not available). In a more recent study (37), we found much higher reproducibility (ICC = 0.62) over a 1-year period when we included only ovulatory women who provided their samples in the midluteal phase of their cycle. Thus, the reproducibility of estradiol levels (in the same phase of the cycle) in premenopausal women needs further evaluation. Intraclass correlations for plasma estrogens from the largest studies to address this issue are provided in Table 3.

Although the data are not entirely consistent, in general, this level of reproducibility (ICC of 0.5–0.8) is similar to that found for other biologic variables, such as blood pressure, pulse, and cholesterol measurements, all exposures that are considered to be reasonably well measured and are consistent predictors of disease in epidemiologic studies (38). Of note, reproducibility data such as these (that measure within-person variation in levels over time) can also be used to explicitly correct for measurement error in studies of plasma hormones and disease risk (39).

Assay and Laboratory Precision

Overview

In contrast to clinical needs, in epidemiologic studies we are generally interested in detecting modest differences within the normal range of hormone levels; laboratory error could easily result in true (and important) associations being missed. This issue has been particularly important in the measurement of plasma estrogens in postmenopausal women, as normal levels are in the picogram per milliliter range and between-person variation in levels is relatively small. Given the limited quantity of plasma collected in most population studies, being able to conduct the assay with a small plasma volume is also important and makes high reproducibility (and high sensitivity) even more difficult to achieve. Another issue is the varying sensitivity and specificity of hormone assays used by different laboratories and, thus, by different epidemiologic studies. These differences make the comparison of findings between published studies difficult.

Reproducibility and Validity of Hormone Assays

Several studies have been conducted to assess the ability of laboratories to reproducibly measure plasma steroid levels in postmenopausal women (40–43). We sent replicate samples of plasma to each of four well-established endocrine laboratories in the United States on one or two separate occasions. All replicate

Table 3. Intraclass correlation coefficients (ICC) and 95% confidence intervals (95% CI) for plasma estrogens in samples collected over a 1- to 3-year period

Hormone	No. of women	ICC	95% CI
Among postmenopausal women*			
Estradiol	79	0.68	(0.59–0.80)
% free estradiol	79	0.80	(0.73–0.87)
% bioavailable estradiol	79	0.86	(0.82–0.92)
Estrone	79	0.74	(0.66–0.83)
Estrone sulfate	79	0.75	(0.67–0.84)
Among premenopausal women			
Estradiol (luteal phase)†	60	0.06	(0.00–§)
% free estradiol‡	71	0.72	(0.57–0.83)
% SHBG estradiol‡	71	0.83	(0.73–0.90)
Estradiol (luteal phase)	39	0.62	(0.43–0.78)
Estradiol (follicular phase)	85	0.53	(0.37–0.67)

*Hankinson et al. (32); three measurements collected over a 2- to 3-year period.

†Muti et al. (35); two measurements collected over a 2- to 3-year period.

‡Toniolo et al. (25); two to three measurements collected over a 2- to 3-year period. SHBG = sex hormone-binding globulin.

§Not provided.

||Two measurements over a 1-year period (37). Luteal phase estradiol was calculated among women with progesterone level ≥ 300 ng/dl who collected each sample 4–10 days before the start of the next cycle.

samples were handled identically during processing, storage, and retrieval, and they were labeled to preclude their identification by the receiving laboratory. The within-person coefficient of variation, a measure of laboratory error frequently reported by laboratories, was consistently low (<15%) for several hormones. For estrone and estradiol, however, hormones present at low levels in postmenopausal women, the laboratory error was often large (>25%), and the ratio of between-person variation to laboratory error was often less than 2.0. Several other studies have also reported variability in assay reproducibility of both plasma (41,42) and urinary (43) steroid hormones, although results depended on the laboratory conducting the assay, the specific hormone, and the menopausal status of the woman.

A number of factors may have influenced the variable reproducibility observed. First, differences in laboratory methods may be important. For example, in our study, although all laboratories used radioimmunoassay (RIA) to measure estradiol, two laboratories used celite column chromatography and one laboratory used LH20 Sephadex column chromatography prior to RIA, whereas the fourth laboratory did not use a separation step prior to RIA. The laboratory method could not have been the only source of error, however, because results also varied within a single laboratory (e.g., the CV for estradiol ranged from 8% to 59%). These substantial differences might relate to a change either in the laboratory personnel or in the reagents and equipment used in the assays or perhaps varying levels of performance by the same technician or piece of equipment over time. In addition, some laboratories may be set up primarily to assay clinical specimens. The level of error tolerable in a clinical setting, in which the distinction between normal and abnormal hormone levels is of primary interest, is substantially greater than that which can be tolerated in epidemiologic research, in which relatively small differences within the spectrum of normal hormone levels are the subject of investigation.

Another technical challenge in studies of hormones is that a number of different laboratory methods are used to measure the same hormone, and no standardization or validation programs exist. For example, in several studies in which plasma estradiol was measured in postmenopausal women, mean levels were 9 pg/mL (44), 13 pg/mL (45), and 28 pg/mL (46). To what degree these differences represent different study populations or simply differences in laboratory methods is unclear and complicates any comparison of results between the studies. The comparison of different laboratory methods against a "gold standard" would be helpful in resolving this issue; however, it is unclear which analytic method would be most appropriate as the gold standard.

Summary and General Recommendations

On the basis of our current knowledge, several recommendations can be made to epidemiologists wanting to use hormone measurements in their research. Close collaboration with laboratory experts should be obtained in the planning stages of a study and should continue through its conclusion. Any variation from the standard collection and processing procedures should be evaluated prior to their implementation. Before having any study blood samples analyzed, laboratory performance should be independently evaluated. After this initial assessment, a proportion of samples sent to the laboratory with each batch of study samples should be quality-control specimens that are indistinguishable from the case and control specimens. Matched case-control pairs should be handled identically and together, shipped

in the same batch, and assayed in the same analytical run. All assays should be conducted without knowledge of the case/control status. Identical handling of all case and control specimens is critical to validity, as any possible deterioration related to collection, processing, or storage should affect case and control specimens equally and will not appreciably affect measures of association. Finally, collection of repeated blood or urine samples from a subset of study subjects should be considered; this collection will allow both the evaluation of within-person variability over time and the use of measurement error correction techniques in the calculation of relative risks.

FOURIER TRANSFORM-INFRARED/STATISTICS MODELS

Fourier transform-infrared (FT-IR) spectra of DNA have revealed major structural differences at various stages in the progression of morphologically normal estrogen-responsive tissues (ERT) to cancer (1-5). Reactions of the hydroxyl radical ($\cdot\text{OH}$) with the base (1-6,47-50) and deoxyribose (1-5) structures have been implicated as major contributors to these modifications, although other factors, to include hypermethylation (51) and the formation of depurinating adducts (52), may modify DNA spectra. In ERT (e.g., the human breast), the $\cdot\text{OH}$ is believed to arise from the metal-catalyzed decomposition of H_2O_2 , which is produced from redox cycling of catechol estrogen metabolites (48) and certain xenobiotics (e.g., aromatic hydrocarbons) (53). The structural differences are evident from wavenumber-by-wavenumber statistical comparisons of the mean FT-IR spectra of DNA (extracted with phenol) (1) from normal and transformed tissues (e.g., normal prostate versus prostate cancer) (5). Principal component analysis (PCA) (4) allows most of the information in each spectrum to be represented by a few principal components (PCs), the first three usually accounting for more than 80% of the total variance. Each PC score is a weighted sum of spectral absorbances. Plots can be constructed on the basis of the first two or three PCs. In these plots, a point represents a single spectrum, and groups (clusters) of points represent the DNA from a particular tissue type (e.g., prostate cancer). In the carcinogenic transformation of one tissue type to another (e.g., normal \rightarrow cancer), the location of the cluster and its diversity in PC space are important measures of DNA change (49). When spectral differences exist between the DNA of tissue groups in a disease progression (e.g., normal tissue \rightarrow cancer), discriminant analysis can be used to establish cancer prediction models, such as those reported for breast (1,4) and prostate (4,5) cancers.

Prototype prediction models, based on multivariate analysis of infrared spectral data, have been developed, and they have an ability to potentially differentiate between tissue groups that were not satisfactorily differentiated by simpler statistical models. These models can potentially distinguish nonmetastatic primary tumors from those with disseminated metastases. Examples of FT-IR/statistics models for predicting cancer-related changes in DNA prior to evidence for cellular transformations are presented, together with discussion of their clinical and etiologic implications.

Significant differences were found between the mean absorbances of DNA from the morphologically normal ovary (O_n) and ovarian adenocarcinoma (AC) over most of the spectral region (Fig. 3, A) (54). The *P* values are presented for each wavenumber (Fig. 3, B). Statistically significant differences (from about 1650 cm^{-1} to 1680 cm^{-1} , 1200 cm^{-1} to 1260 cm^{-1} , and 1000 cm^{-1} to 1150 cm^{-1}) are evident in spectral areas assigned to

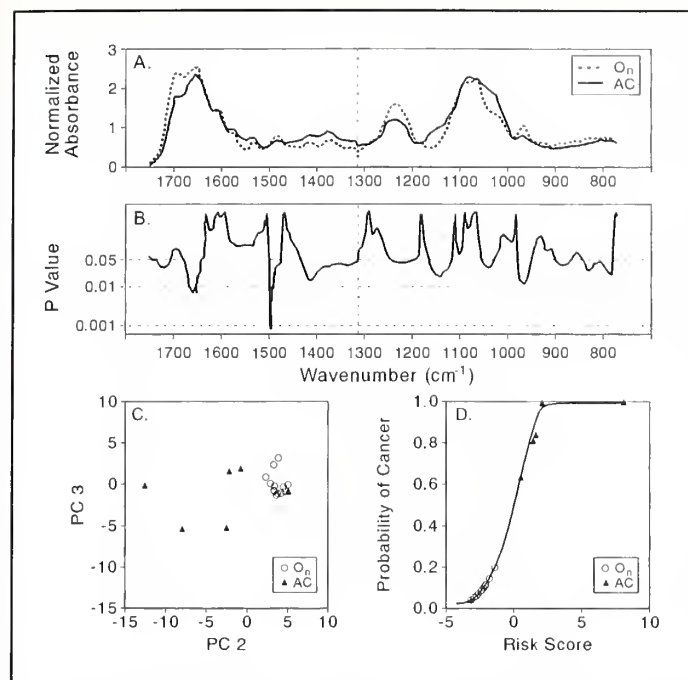


Fig. 3. Spectral comparisons of ovarian DNA (53). **A)** Grand mean DNA spectra of morphologically normal ovarian tissue (O_n; n = 13) and primary ovarian adenocarcinoma (AC; n = 6); **B)** P values for spectral comparison in **A** (unequal variance *t* test) (53); **C)** principal component (PC) plot comparing spectra of ovarian DNA from morphologically normal tissues (O_n) and primary adenocarcinoma (AC) (53); and **D)** plot of the probability of ovarian cancer with the risk score for the O_n and AC. The null hypothesis that the PC scores do not discriminate between the groups is rejected with *P* < .001. Reprinted with permission © 1998 from the National Academy of Sciences, U.S.A.

vibrations of the nucleotide bases, the PO₂⁻ group and deoxyribose, respectively (54). PCA of the spectral data provided two major PCs that were plotted against each other (Fig. 3, C). The plot revealed that the O_n formed a tight, ordered group of points,

whereas the ACs were highly diverse and relatively disordered. The relationship between the probability of ovarian cancer and the risk score derived by discriminant analysis is shown in Fig 3, D. The ovarian cancer group is located primarily at the top of the sigmoid-like curve, and the noncancer group is located at the bottom. The predicted probability scores rise rapidly over a narrow range, which reflects a high degree of discrimination between the groups. The disorder reflected in the AC and the metastasized primary ovarian adenocarcinomas (AC_m) contrasts with the order in the O_n and distant ovarian metastases to the colon (AC_{dm}), as apparent from the mean spectral comparisons (Fig. 4, A) and the PC plot in which the points of each group substantially overlap (Fig. 4, B) (54). Despite the inability to discriminate between the two ordered DNA systems with the use of spectral comparisons and PCA, comparisons of standard deviations of absorbances at each wavenumber over the entire spectral range revealed increased spectral diversity in the AC_{dm} in regions assigned to base vibrations but not in those relating to the furanose ring. This finding is consistent with the presence of increased base mutations in the DNA of the distant metastases (54).

Comparisons of the mean spectra of DNA from morphologically normal breast tissues obtained from breast reduction surgery (reduction mammoplasty tissues, RMT) and invasive ductal carcinoma (IDC) tissues revealed characteristic differences in spectral regions assigned to the base and deoxyribose structures (1–4). A three-dimensional plot of the points from PCA is given in Fig. 5, A. The points representing the RMT are clustered primarily in the upper-left region of the plot. The IDC, comprising primary tumors with and without evidence for axillary metastases, are broadly dispersed, thus indicating considerable structural diversity. Discriminant analysis of the spectral data provided a relationship between the probability of cancer and the risk score (Fig. 5, B), having a sensitivity of 86% and a specificity of 81%. The DNA of “benign” (microscopically normal) tissues from near the breast tumors of 11 women (not included

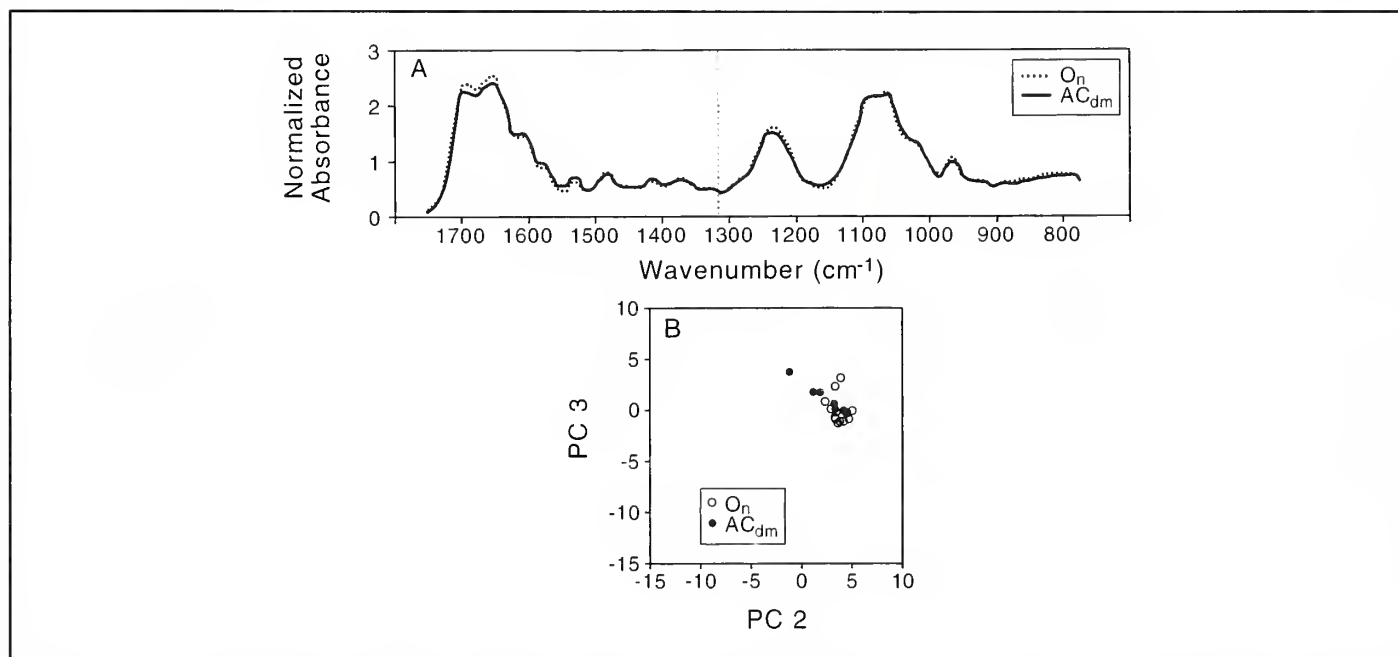


Fig. 4. Spectral comparisons of ovarian DNA (53). **A)** Mean spectra of DNA from the morphologically normal ovary (O_n; n = 13) compared with mean spectra of DNA from ovarian adenocarcinoma metastases to the colon (AC_{dm}; n = 7); **B)** principal components plot comparing the mean DNA spectra from each group shown in **A**. Reprinted with permission © 1998, National Academy of Sciences, U.S.A.

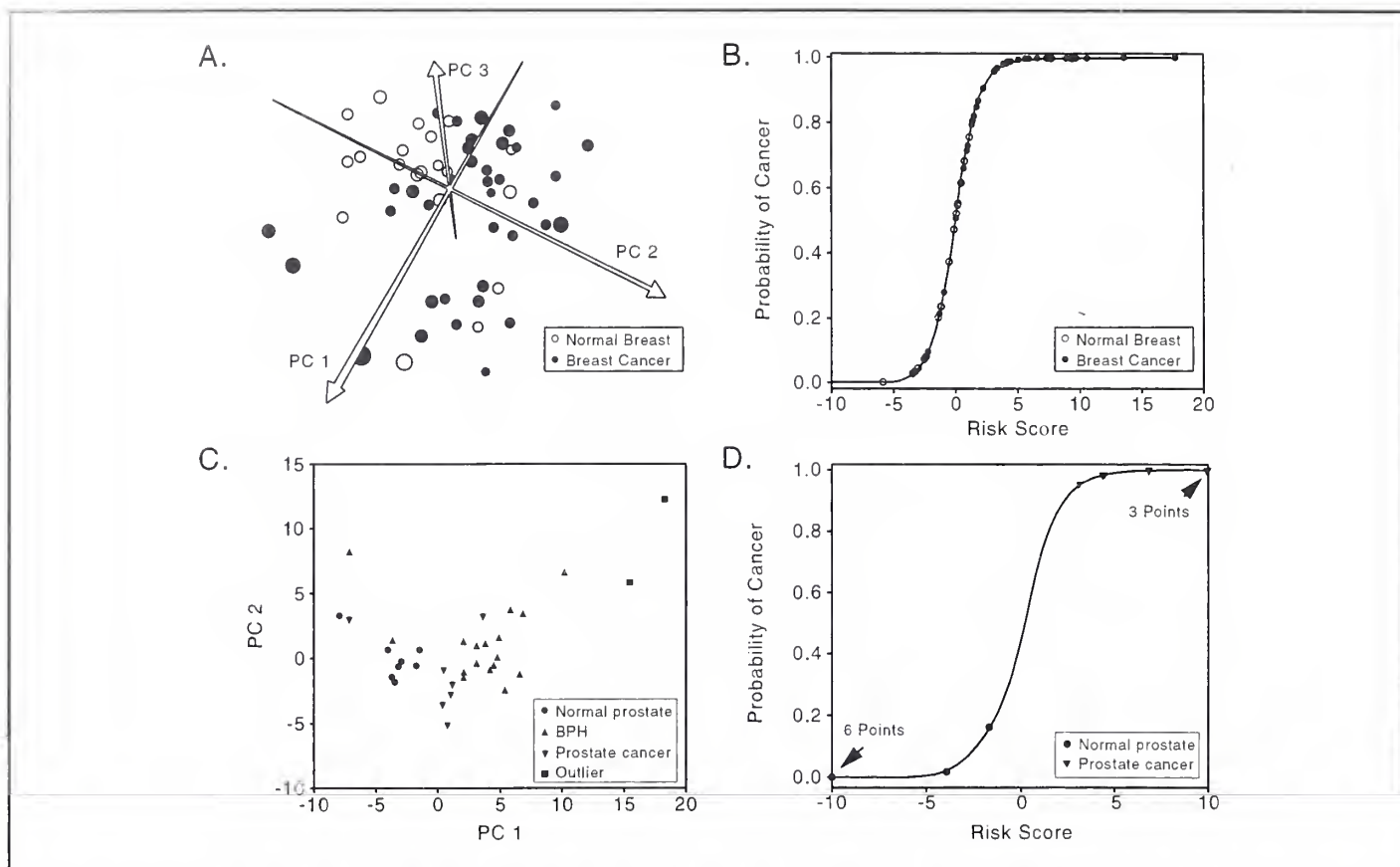


Fig. 5. Spectral comparisons of breast DNA (4). **A)** Three-dimensional plot of principal component (PC) scores of DNA spectra from normal breast ($n = 21$) and breast cancer (invasive ductal carcinoma [IDC]; $n = 37$) tissues showing distinct clustering of each group (1,3); **B)** plot of the probability of cancer with the risk score for the normal breast and breast cancer. The null hypothesis that the PC scores do not discriminate between the groups is rejected with $P < .0001$ (1); **C)** two-dimensional plot of PC scores of DNA spectra from normal prostate

($n = 8$), benign prostatic hyperplasia (BPH; $n = 18$) and prostate cancer (adenocarcinoma; $n = 8$) in which the clustering is distinct (5); **D)** plot of the probability of cancer versus the risk score for normal prostate and prostate cancer. The null hypothesis that the PC scores do not discriminate between the groups is rejected with $P = .009$ (5). Reprinted with permission by *Nature Medicine*. Portions A and B originally appear in *Nat Med* 1997;3:927–30.

in the predictive model) was analyzed (4). When the scores were used in the breast cancer probability-risk score model (Fig. 5, B), 10 of 11 had a predicted cancer probability of more than 75%. This finding is consistent with data showing that tissue near a breast tumor has a high risk for forming a second cancer lesion (55).

In studies of the human prostate, the mean spectral differences between the DNA of normal tissue and the DNA of prostatic adenocarcinoma were substantial. The PC plot revealed pronounced discrimination between DNA spectra of normal and cancer tissues (4,5). A similarly effective separation was obtained between the clusters of DNA points representing normal tissue, prostatic cancer, and benign prostatic hyperplasia (BPH) (Fig. 5, C). The discriminant analysis models that predict disease probability (normal prostate tissue versus prostate cancer; normal prostate tissue versus BPH) had sensitivities and specificities of 100% for both comparisons. These models are based on more PC scores than the two-dimensional PC plots. The relationship between the normal prostate DNA and the DNA of prostatic adenocarcinoma, expressed in terms of cancer probabilities, is shown in Fig. 5, D.

Prototype statistical models, based on FT-IR spectroscopy, are being tested in our laboratory. These models hold promise for distinguishing the DNA from primary tumors and metastasizing primary tumors (those that have given rise to dissemi-

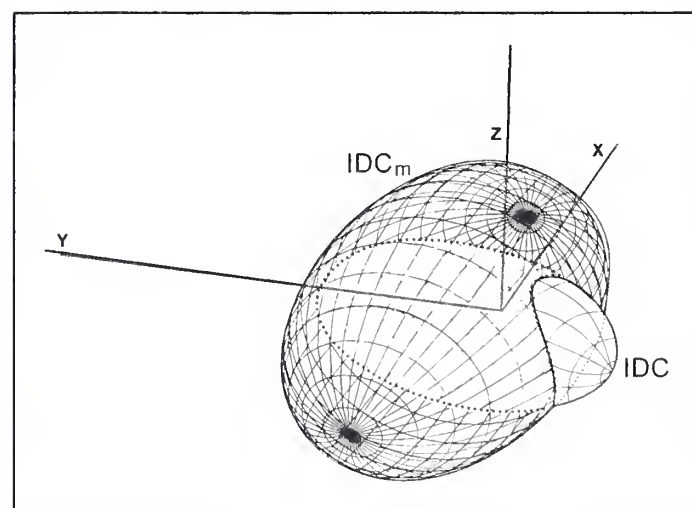


Fig. 6. Three-dimensional ellipsoids based on a multivariate model for principal component (PC) scores of DNA spectra of invasive ductal carcinoma (IDC) and metastasized IDC (those that give rise to disseminated metastases; IDC_m). The ellipsoids contain an expected 90% of the population of each group (e.g., IDC samples). (See text for details.)

nated metastases). The FT-IR/statistics models based on simple linear logistic regression, such as those shown in Fig. 5, B, did not effectively differentiate these groups. By use of models based on multivariate normal distributions of the first three PCs, a three-dimensional projection (Fig. 6) was constructed to contain a designated percentage (i.e., 90%) of the population of a group. In a model with 90% probability, such as that shown in Fig. 6, a randomly selected IDC_m spectrum would likely fall inside the appropriate three-dimensional figure (i.e., only an expected 10% of DNA spectra in the population of IDC_m spectra would fall outside the model). By use of this DNA model, normal breast tissue (RMT), primary breast tumors (IDC), and metastasizing primary breast tumors (IDC_m) were correctly classified as follows: 89% (16 of 18), 97% (31 of 32), and 82% (18 of 22), respectively. The discrimination between the IDC and the IDC_m is a potentially important basis for identifying metastasis in primary tumors, prior to evidence for malignant cells at distant sites. The prototype model (Fig. 6), which is presently based on a limited number of samples, can be applied to other systems having larger databases.

The FT-IR/statistics models have the ability to identify subtle changes in DNA in relation to the progression of normal tissues to diseased states. We are unaware of other techniques with the power to accomplish such a high degree of discrimination between DNA of natural systems. It is now possible to analyze less than 1.0 µg of DNA with the use of FT-IR spectral techniques recently developed in our laboratory. This will eventually allow the FT-IR/statistics technology to be applied to less than 1.0 mg of tissue, thus broadening the application to small biologic samples (e.g., fine-needle biopsy tissues). Future uses of the technology would be expected to encompass diverse areas of cancer research and clinical practice, as previously described (4). For example, with the use of the FT-IR/statistics technology, the potential exists for detecting early cancer-related changes at the level of DNA, rather than at the cellular level, thereby affording a distinct advantage in patient treatment.

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NOTES

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Chapter 10: Hope for Prevention—Perspective of the Cancer Advocate

Elizabeth A. Hart

Breast and prostate cancer survivors and advocates participated as panelists with scientists in an interactive panel discussion following 2 days of scientific presentations on "Estrogens as Endogenous Carcinogens in the Breast and Prostate." Advocates raised several issues of concern and questions related to the research presented. Concerns included the following: 1) a global fear of developing either breast or prostate cancer and recurrence from these tumors, 2) a specific fear that estrogen replacement therapy could enhance the development of new breast cancers and stimulate recurrence in breast cancer survivors, and 3) a concern that researchers examining minority communities should have sensitivity to the specific culture under study and an understanding of specific research issues that are relevant in those communities. The questions raised included the following: 1) What are the implications of resistance to antiestrogen therapies and what is the appropriate sequencing of hormone therapy for longer-term benefit? 2) Can one identify women and men at risk for cancer who do not have the usual risk factors? 3) Where does the development of blood or urine tests to screen for cancer currently stand? 4) Can research findings be translated into effective therapies more rapidly? 5) Can the status of this translational process be communicated to the public in a meaningful way by breaking down language barriers? 6) What means are available to develop more creative ways to fund pilot studies that do not require preliminary data and to create new funding mechanisms to respond to the needs of scientists, particularly those that work collaboratively from multiple institutions and multidisciplines? 7) How can the need for increased emphasis on and visibility for prostate cancer be communicated? Following the interactive dialogue, scientists and advocates suggested more collaborative research with sustained funding avenues, continued dialogue and collaboration between scientists and advocates, and more collaborative research groups like the Cancer Cube. [J Natl Cancer Inst Monogr 2000;27:157-9]

COMPOSITION OF PANEL

A multidisciplinary panel, chosen to create a dialogue between scientists and patient advocates, discussed concepts arising from 2 days of intense scientific discussion at a meeting held on March 16-17, 1998, entitled "Estrogens as Endogenous Carcinogens in the Breast and Prostate." This landmark meeting was convened by a collaborative group called "The Cancer Cube," which is interested in demonstrating the causes of breast and prostate cancer. Panelists included the following:

- a young breast cancer survivor diagnosed at age 38 years,
- a breast cancer survivor with metastatic disease,
- a prostate cancer survivor with a brother and father (deceased) diagnosed with the disease,
- a prostate cancer advocate charged with the development of

prostate cancer support groups nationwide and advocacy on behalf of those with prostate cancer,

- a high-risk individual with multiple family members either deceased or surviving breast, prostate, kidney, uterine, throat, and lung cancer,
- a scientist expert in chemical carcinogenesis,
- a scientist expert in clinical/translational sciences, and
- a scientist expert in hematology, oncology, and endocrinology.

In addition, all scientists (both presenting and in the audience) and advocates participated in an interactive dialogue following brief presentations by panel members. Advocates on the panel listened to the research presented for 2 days and then articulated issues important to the advocacy community and to the public at large relating to the presentations.

QUESTIONS AND CONCERNS

The issue of cancer prevention is the most critical concern to breast and prostate cancer survivors, their families, advocates, and the public at large. The rapid advances in cancer research in recent years raise expectations that an answer may well be forthcoming in the not-too-distant future. For some, the answer will come too late. Within the advocacy community and the public, there is a tremendous sense of urgency to advance research from the laboratory to the clinical setting as quickly as possible. Fear was an underlying theme throughout the discussion: fear of cancer in general and fear of recurrence. A specific fear related to the possibility that estrogen replacement therapy could increase the risk of developing new breast cancers and the rate of recurrence in breast cancer survivors (1).

IMMEDIATE BENEFITS OF CURRENT RESEARCH

Recent development of "designer antiestrogens" and use of surrogate drugs to give the benefits of estrogen without using estrogen itself were considered to be important practical advances. These approaches could immediately benefit patients. Designer estrogens that act positively to reduce bone loss, subsequent osteoporosis, and bone fractures, and yet do not adversely affect the breast, are now available (1). The potential of these agents to prevent cardiovascular disease is currently under study.

Following in this same vein are compounds that are being developed and tested as new antiestrogens. The potential is for longer-term benefit to women with metastatic breast cancer whose hormone-dependent tumors have become resistant to the standard antiestrogens (1). Blocking estrogen production with

Correspondence to: Elizabeth A. Hart, R.N., B.A., Hart International, 9051 Oak Path Lane, Dallas, TX 75243 (e-mail: hart.elizabeth@worldnet.att.net).
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aromatase inhibitors, such as anastrozole (Arimadex) and letrozole (Femara), continues to provide benefit to patients whose tumors are resistant to standard antiestrogens. "Pure" antiestrogens, which do not have the potential to exert estrogen-like effects on certain tissues, are under study. These agents appear to be promising for the treatment of women whose breast tumors are resistant to tamoxifen. It has been suggested that some combination of these therapies, tailored to the individual's particular parameters, can extend the benefits of hormonal therapy.

Quality of life is an important issue for women surviving breast cancer. A number of treatments are currently available to alleviate estrogen-deficiency symptoms experienced by these women and to serve as surrogates for estrogen. As examples, the bisphosphonates act to prevent osteoporosis, the statin drugs lower cholesterol and ultimately prevent heart disease, low-dose vaginal estrogens provide relief from urogenital atrophy, and antidepressants deal with depression exacerbated by estrogen deficiency in susceptible individuals (1).

For prostate cancer survivors who experience medical or surgical castration and have vasomotor instability, many have benefited from the administration of Clonidine or Megace. Again, the bisphosphonates are useful in treating osteoporosis in settings where androgens and estrogens are deficient (2).

INTERMEDIATE BENEFITS OF CURRENT RESEARCH

Research presented offering intermediate benefits (research in process and not yet available for immediate application) to breast and prostate cancer survivors involves the continuing development of new drugs acting as hormonal antagonists. Some of these work specifically on the β -estrogen receptor and might have beneficial estrogen effects without having detrimental effects on the breast. For the prostate, researchers are beginning to look at the use of aromatase inhibitors in men with advanced prostate cancer. This approach is based on the hypothesis that there are mutations of the androgen receptor in advanced prostate cancer that make the receptors promiscuous in the sense that they are stimulated to a greater extent with estrogen than with androgen (1).

LONG-TERM BENEFITS OF RESEARCH

For the long term, the hope is to prevent breast and prostate cancers. Greater understanding of the metabolic activation of estrogens in the body may suggest potential new prevention strategies. Scientists at the meeting suggest that certain metabolites of estrogen, both exogenous and endogenous, can generate mutations that could lead to cancer (Chapters 3 and 4). The theory suggests that estrogen receptor-mediated processes would allow these mutations to be propagated. Estrogens acting through receptors could induce cellular proliferation and increase the replication of mutated genes. Together, the genotoxic and cell proliferative effects of estrogen would enhance the process of cellular transformation and, eventually, cause cancer (*see Symposium Overview*).

Metabolic activation of estrogens involves the formation of catechol estrogen metabolites (products of estrogen metabolism, Chapter 5), which, when oxidized in a specific pathway, bind to DNA. These DNA-estrogen complexes cause depurination of DNA (adenine and guanine bases that fall out of DNA) and other DNA damage that leads to tumor initiation (Chapter 4). There is mounting evidence that the pathway leading to the formation of 4-hydroxy estrogens (carcinogenic in animals) is the real culprit,

particularly when the enzymes (catechol-*O*-methyltransferases) that normally neutralize these products of estrogen metabolism are not present or are present at very low levels and, therefore, are not effective protectors (3–6). Of interest, the 2-hydroxylated estrogens, the major products of estrogen oxidation in mammalian species, form stable DNA adducts and are not carcinogenic (3–5). If these concepts are borne out, both breast and prostate, as well as other cancers, would share the same initiation process. The implications for prevention are immediate: inhibit metabolic activation of estrogens (particularly to 4-hydroxy estrogens) and enhance their metabolic protection (3,4).

RISK FACTORS

Another issue of significance to advocates was the ability to identify women early on who do not have the usual risk factors. It was considered important as well to utilize blood or urine tests to screen for cancer. There are currently studies in process looking at biomarkers of DNA damage prior to the development of breast cancer, one of which is a blood assay indicating the presence of high anti-HmdU (5-hydroxymethyl-2'-deoxyuridine: an oxidized thymidine) autoantibody titers in healthy women with a family history of breast cancer (7). This would permit the screening of individuals prior to the clinical manifestation of cancer and provide the possibility of prevention.

HORMONE RESISTANCE

Resistance to antiestrogen therapy and the appropriate sequencing of hormone therapy for longer-term benefit was discussed at length as a major issue. Clearly, there is benefit to complete blockage of estrogen in individuals relapsing on tamoxifen (Nolvodex) in some settings, yet, in other settings, it is inappropriate to begin with complete estrogen blockade and then expect an antiestrogen to work effectively. So, the most effective sequence for maximum benefit over time seems to be the treatment with adjuvant antiestrogens that are strong antagonists but weak agonists, followed later on by a pure antiestrogen (1).

TRANSLATION

In addition to the specific research issues raised, advocates focused on translating research findings to the public in a meaningful way. How could one break down the language barrier between scientists, advocates, and the public at large? Advocates have become increasingly involved in the research process in the last 10 years, having successfully led the push for increased funding for cancer research, having participated in peer review of basic, clinical, and translational research proposals [(8); Andejski Y, Sharp-Breslau E, Hart E, Lythcott N, Alexander L, Rich I, et al.: manuscript in preparation for publication], and are increasingly included on policy, review, and decision-making bodies. They read the scientific literature. Many have large constituencies in need of accurate research information that is readily available in layman's terms and can be easily disseminated to the public. Scientists were urged to begin to break through the language barrier with the idea of making research findings readily accessible to the lay individual in terms that they could understand. Advocates want to be involved and to help in moving research forward on the fastest track possible, including assistance with funding.

FUNDING OF RESEARCH

Funding of research was a major point of discussion. Advocates felt that more creative ways of funding pilot studies should be forthcoming. Funding of those studies should not necessarily require preliminary data. At the moment, limited ways of funding exist for pilot studies that can subsequently lead to applications for more traditional funding. Collaborative efforts such as the Cancer Cube (*see* Introductory Remarks) have an equally difficult time finding appropriate funding mechanisms, although efforts are being made by the National Cancer Institute (Bethesda, MD) to address this issue. Members of the Cancer Cube, which include an advocate, work in multiple research centers. Collaboration among centers provides a unique way to enhance research because it allows wide sharing of specific expertise and cutting-edge technology. Highly technical resources are shared, and specific objectives are chosen by the group to advance research more efficiently and effectively.

Funding of research on prostate cancer remains definitively lower than that of breast cancer or acquired immunodeficiency syndrome (AIDS). In the 1997/1998 Labor HHS Appropriation, signed in November 1997, prostate cancer received approximately \$89.5 million compared with \$348.6 million in breast cancer, with AIDS at \$226.4 million (9). Prostate cancer needs more visibility, as do a number of other cancers. However, the advocates and scientists both felt strongly that one disease should not be pitted against another. The need is to figure out how to raise sufficient monies to fund the necessary basic, clinical, and translational research which, in specific instances, has applicability in a number of diseases, not just breast or prostate cancer. It is important as well to have trained researchers from and in minority communities, who have sensitivity to the culture and understanding of specific research issues that are relevant in those communities. The U.S. Department of Defense is currently conducting a consensus conference strategy to develop a blueprint for including training of minority researchers at the predoctoral through postdoctoral level.

CONCLUSION

The interactive dialogue between scientists and advocates was bold and enlightening, with articulation from both groups as to needs, cooperative efforts, and future directions. Advocates suggested more collaborative "Cubes" and creative funding mechanisms, while scientists suggested more collaborative research with sustained funding avenues and continued dialogue and collaboration with advocates. It was expressed and abundantly clear that both advocates and scientists share an equal

passion for finding a cure and, even more important, preventing the initiation of breast and prostate cancers, as well as other cancers.

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NOTES

The Panel Members include the following: *chair*—Elizabeth A. Hart, President & CEO, Hart International, Dallas, TX; *moderator*—David G. Longfellow, Ph.D., Chief, Chemical & Physical Carcinogenesis Branch, Division of Cancer Biology, National Cancer Institute (NCI), Bethesda, MD; *panel members*—Winston Dyer, Community Coordinator of Minority Clinical Trials, CaP CURE, New York, NY; Carol Hochberg, Board Member and Committee Chairman of the Advocacy and Public Policy Committee of SHARE, New York, NY; Edison Liu, M.D., Ph.D., Director of Clinical Sciences, NCI; M. Brooke Moran, Director of Patient Advocacy and Government Affairs for the American Foundation for Urologic Disease, Baltimore, MD; Diana Rowden, Chairman of the Board of the Susan G. Komen Breast Cancer Foundation, Dallas; and Richard Santen, M.D., Professor, Division of Hematology, Oncology and Endocrinology, University of Virginia Health Science Center, Charlottesville.

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Introduction

Ellen G. Feigal, Judith E. Karp

Scientists, clinicians, health care workers, and community and patient advocates from around the world gathered in May 1999 at two major conferences focused on acquired immunodeficiency syndrome (AIDS)-associated cancers, i.e., the Third National AIDS Malignancy Conference and the International Symposium on HIV, Leukemia, and Opportunistic Cancers. The Third National AIDS Malignancy Conference, sponsored by the National Cancer Institute, was held at the National Institutes of Health campus in Bethesda, MD. The International Symposium on HIV, Leukemia, and Opportunistic Cancers, sponsored by the International Association for Comparative Research on Leukemia and Related Disorders (IACRLRD) and the Harvard AIDS Institute and cosponsored by the Leukemia Society of America, the International AIDS Society, and the Pasteur Institute of Morocco, was held in Morocco. This monograph contains a selection of the papers presented at these two multidisciplinary meetings.

AIDS, caused by the retrovirus known as human immunodeficiency virus (HIV), was first recognized two decades ago. Since that time, scientists have made much progress in understanding the etiology, pathogenesis, and pathophysiology of this devastating disease. The study of AIDS has generated important concepts that apply broadly to stem cell biology and immunobiology. In turn, these basic scientific discoveries have served as a springboard for exciting therapeutic advances that hold great promise with respect to deterring, reversing, and, in some cases, preventing progressive HIV disease.

AIDS remains a major killer throughout the world. A particular challenge is the link to certain types of malignancies, driven at least in part by the prolongation of survival in the face of impaired immunity. The molecular and clinical dissection of these complex diseases is highly instructive with regard to settings where immunodeficiency promotes or permits tumorigenesis—for instance, post-transplant lymphoproliferative disorders. The entire area of viral oncogenesis continues to be informed by AIDS-related malignancies, in particular the tumorigenic roles of Epstein-Barr virus and human papillomaviruses,

and the etiologic relationship of human herpesvirus 8 to disparate malignancies such as Kaposi's sarcoma (KS) and primary effusion lymphomas. In turn, these malignancies provide a pivotal testing ground for antiviral and immunomodulatory strategies, including antiangiogenesis and cytokine-based approaches, adoptive immunotherapy, vaccine development, and gene-based strategies that could have a major impact on a broad spectrum of cancers.

Ultimately, however, the major goal of AIDS research is to prevent progressive HIV infection and HIV-induced immunosuppression. The impact of multitargeted, highly active antiretroviral therapy is already being felt in terms of AIDS-associated KS. Moreover, the ability to preserve and, in some instances, to induce regeneration of a competent immune system stands to have a future impact on the incidence of virally induced lymphoproliferative diseases and virally related epithelial tumors. Most importantly, the ability to deliver the current and future advances to the entire global population is essential to eradication of the worldwide devastation that continues to result from AIDS infection.

The next conference focused on AIDS malignancy is the Fifth International AIDS Malignancy Conference, which will take place on April 23–25, 2001, in Bethesda, MD. It will include sessions on human papillomavirus, Epstein-Barr virus, and Kaposi's sarcoma-associated herpesvirus. Registration and other conference information are available on the website <http://ctep.info.nih.gov/AIDSONcoResources>. A summary of the Fourth International AIDS Malignancy Conference is available on the website <http://hiv.medscape.com/conferences/malignancy2000>.

Affiliations of authors: E. G. Feigal, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD; J. E. Karp, Greenebaum Cancer Center, University of Maryland, Baltimore.

Correspondence to: Ellen G. Feigal, M.D., National Institutes of Health, Bldg. 31, Rm. 3A44, Bethesda, MD 20892.

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Kaposi's Sarcoma in South Africa

Freddy Sitas, Robert Newton

Kaposi's sarcoma was endemic in South Africa even before the advent of the human immunodeficiency virus (HIV). Between 1988 and 1996, the incidence of Kaposi's sarcoma in South Africa has risen at least threefold and continues to increase as the HIV epidemic grows. Research from South Africa has shown that infection with human herpesvirus 8 (HHV8) is associated with Kaposi's sarcoma but not with any other major cancer site or type. In addition, the risk of Kaposi's sarcoma increases with increasing antibody titer to HHV8, but, for a given titer, the risk is greater in HIV-seropositive compared with HIV-seronegative individuals. The age- and sex-standardized seroprevalence of HHV8 in black South African hospital patients was found to be slightly more than 30%; the seroprevalence of HHV8 increased with age and was similar in men and in women. The modes of transmission of HHV8 are yet to be fully elucidated. Limited evidence exists for sexual transmission in black South African adults, but mother-to-child and person-to-person transmission in childhood is also likely. Furthermore, the seroprevalence of HHV8 decreases with increasing levels of education and is lower in whites than in blacks, suggesting that factors associated with poverty may be important determinants of transmission. Future research should focus on risk factors for Kaposi's sarcoma in HHV8-infected individuals, on determinants and mode of transmission of HHV8, and on the elucidation of the effect of primary HHV8 infection in adults and in children. [J Natl Cancer Inst Monogr 2000;28:1-4]

Before the human immunodeficiency virus (HIV) epidemic, Kaposi's sarcoma showed a greater geographic variation in incidence than almost any other cancer. It was as common in parts of sub-Saharan Africa, such as Uganda and eastern Zaire, as colon cancer is in Europe and the United States, representing up to 9% of all cancers in men (1-4). Narrow belts of relatively high incidence stretched westward across the former Zaire to the coast of Cameroon and southward down the Rift Valley into Malawi and parts of South Africa (Fig. 1) (4,5). Kaposi's sarcoma was also endemic, although much rarer, in countries around the Mediterranean, particularly Italy, Greece, and the Middle East, but it was almost nonexistent elsewhere in the world, except in immigrants from those endemic countries (6-8). In all of these areas, Kaposi's sarcoma was considerably more common in men than in women (4).

HIV AND KAPOSI'S SARCOMA

It was the appearance of aggressive forms of Kaposi's sarcoma in the United States in the early 1980s that heralded the onset of the HIV epidemic in western countries. Although the incidence of Kaposi's sarcoma has increased in populations at high risk of HIV in northern Europe and in the United States, it existed in the rest of these populations at such a low level before the onset of the epidemic that it still remains a relatively rare tumor (6,9). However, parts of Africa with a high prevalence of

HIV and where Kaposi's sarcoma was relatively common even before the era of acquired immunodeficiency syndrome (AIDS) have seen an explosion in the incidence of the disease. In the past 10-15 years, the incidence of Kaposi's sarcoma has increased about 20-fold in Uganda and Zimbabwe, such that it is now the most common cancer in men and the second most common in women (10,11). Similarly, between 1988 and 1996, the incidence of Kaposi's sarcoma has risen at least threefold in South Africa and continues to increase as the HIV epidemic grows (12). Data from the South African National Cancer Registry show that, between 1992 and 1996, the incidence rates of Kaposi's sarcoma have doubled in men but have increased about sevenfold in women, such that the sex ratio of 7:1 in males versus females in 1988 has now declined to only 2:1 (12).

The epidemiology of HIV-associated Kaposi's sarcoma varies around the world, reflecting to a certain extent the situation that existed in the era before AIDS. For example, in a South African study (13), the relative risk of Kaposi's sarcoma in HIV-infected individuals, compared with HIV-uninfected individuals, was 62 (95% confidence interval [CI] = 20-194). Although this is similar to results from elsewhere in Africa (14,15), it is an order of magnitude lower than would be expected from studies in, for example, the United States (5). This result simply reflects the fact that Kaposi's sarcoma is endemic in Africa with a relatively high proportion of HIV-uninfected cases; thus, the absolute risk of developing Kaposi's sarcoma among those individuals who are co-infected with HIV and with human herpesvirus 8 (HHV8) is probably about the same as in the United States.

HHV8 AND KAPOSI'S SARCOMA

HHV8, a newly discovered human herpesvirus (16), has been consistently associated with Kaposi's sarcoma and is now considered to be the principal cause of the disease (17). Genomic sequences of HHV8 are present in tumor cells of Kaposi's sarcoma lesions in virtually all subjects (18), and its presence, detected by polymerase chain reaction or serology in peripheral blood, predicts the subsequent development of the tumor (19,20). Furthermore, HHV8 is not a ubiquitous virus but is most prevalent in groups or populations at highest risk of developing Kaposi's sarcoma, such as HIV-infected homosexual men in the United States and African populations in whom the tumor has long been endemic (4,21,22).

Affiliations of authors: F. Sitas, National Cancer Registry and Cancer Epidemiology Research Group, Department of Anatomical Pathology, South African Institute for Medical Research, University of the Witwatersrand, Johannesburg; R. Newton, Cancer Epidemiology Unit, Imperial Cancer Research Fund, The Radcliffe Infirmary, Oxford, U.K.

Correspondence to: Freddy Sitas, D. Phil., National Cancer Registry and Cancer Epidemiology Research Group, Department of Anatomical Pathology, South African Institute for Medical Research, University of the Witwatersrand, P.O. Box 1038, Johannesburg, 2000, South Africa (e-mail: freddys@mail.saimr.wits.ac.za).

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Kaposi's sarcoma in Africa - pre-1980
Estimated cumulative incidence
males aged 0-64

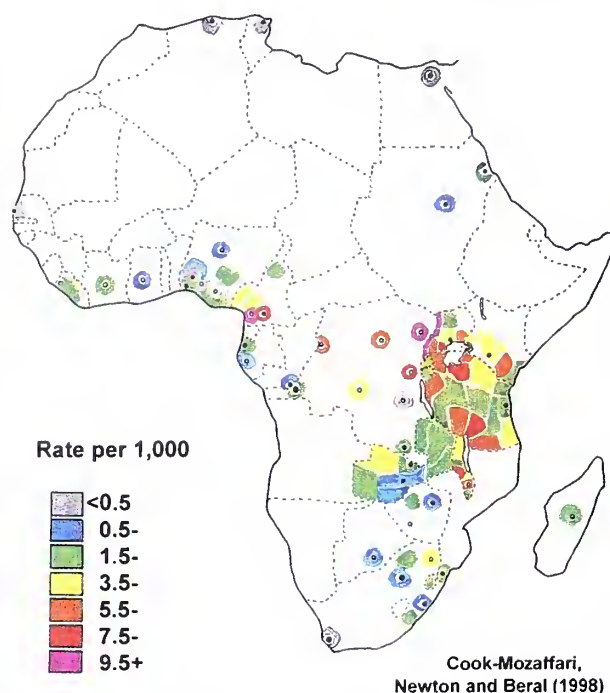


Fig. 1. Estimated cumulative incidence of Kaposi's sarcoma in males aged 0-64 years in Africa, before 1980. Adapted from (5).

A recent case-control study (23) of black cancer patients from Johannesburg and Soweto, South Africa, found that infection with HHV8 was strongly associated with Kaposi's sarcoma but not with any other major cancer site or type, including prostate cancer and multiple myeloma. In addition, the risk of Kaposi's sarcoma increased with increasing antibody titer (as measured by the intensity of the fluorescent signal) to HHV8; however, for a given titer, the risk was much greater in HIV-seropositive than in HIV-seronegative subjects (Table 1). The highest fluorescent signal intensity for HHV8, corresponding to an antibody titer of 1:204 800, was associated with a 12-fold increase in risk of Kaposi's sarcoma among HIV-seronegative subjects but with a more than 1600-fold increase in risk among HIV-seropositive subjects. HHV8 seroprevalence rates and antibody titers to HHV8 were not, however, markedly related to HIV infection among those without Kaposi's sarcoma (Table 2).

Little data on the relation between HHV8 antibody titer and viral load are available, but, presumably, a high anti-HHV8 antibody titer reflects a high viral load of HHV8 and that it is this high viral load, rather than antibody titer, that primarily determines the risk of Kaposi's sarcoma. The excess risk of Kaposi's sarcoma in HIV-seropositive individuals compared with HIV-seronegative individuals may mean that, for a given anti-HHV8 antibody titer, the viral load is higher in those co-infected with HIV than in those who are not co-infected with HIV. Alternatively, high-titer HHV8 infection could reflect a high level of expression of an antigenically important gene product. Nevertheless, the relation between high antibody titer and disease is reminiscent of the association between Epstein-Barr virus (a

Table 1. Relation of Kaposi's sarcoma to fluorescent signal intensity (a measure of antibody titer) for HHV8, according to HIV serostatus*

Fluorescent signal intensity for HHV8 (and median titer)	No. with Kaposi's sarcoma	No. without Kaposi's sarcoma	Odds ratio† (95% confidence interval)
HIV-1-seronegative subjects			
Absent (<1:100)	5	1990	1.0‡
Low (1:200)	2	665	1.5 (0.3-7.8)
Medium (1:51 200)	4	331	6.2 (1.6-24.2)
High (1:204 800)	2	131	12.0 (2.1-68.2)
Test for trend			$\chi^2_1 = 11.4; P = .0007$
HIV-1-seropositive subjects			
Absent (<1:100)	5	105	10.8 (2.9-40.6)
Low (1:200)	2	31	48.1 (7.7-300)
Medium (1:51 200)	10	28	62.2 (18.0-214)
High (1:204 800)	21	8	1682 (390-7253)
Test for trend			$\chi^2_1 = 37.2; P = <.00001$

*HHV8 = human herpesvirus 8; HIV = human immunodeficiency virus.

†Adjusted for age, sex, and, where possible, education and number of sexual partners.

‡Comparison (referent) group.

Adapted from (23).

Table 2. Relation of HIV-1 serostatus to fluorescent signal intensity for HHV-8 in subjects without Kaposi's sarcoma*

Fluorescent signal intensity for HHV8	HIV-1 seropositive	HIV-1 seronegative	Odds ratio† (95% confidence interval)
Absent (<1:100)	105	1990	1.0‡
Low (1:200)	31	665	1.1 (0.6-2.0)
Medium (1:51 200)	28	331	2.0 (1.3-3.2)
High (1:204 800)	28	131	2.0 (0.9-4.4)

*HIV = human immunodeficiency virus; HHV8 = human herpesvirus 8.

†Odds ratio for HIV-1 seropositivity compared with HIV-1 seronegativity, adjusted for age, sex, education, and number of sexual partners.

‡Comparison (referent) group.

Adapted from (23).

related gamma herpesvirus) and African Burkitt's lymphoma (24,25) and nasopharyngeal cancer (26), in which individuals with high titers appear to be at highest risk of developing these cancers.

EPIDEMIOLOGY AND TRANSMISSION OF HHV8 IN SOUTH AFRICA

The modes of transmission of HHV8 are yet to be fully elucidated. In the United States, sex between men may be an important route of transmission because this is the main behavioral risk factor for Kaposi's sarcoma and indeed some evidence now exists that this is so (26). There is weak evidence of sexual transmission of HHV8 in the South African population, although the increase in risk with increasing number of sexual partners was not great (23). Furthermore, no difference was seen in the seroprevalence of HHV8 in those individuals with or without HIV infection. However, throughout sub-Saharan Africa, where Kaposi's sarcoma was seen in children even before the advent of AIDS, other routes of transmission must also be occurring.

In three South African studies (23,27,28), the seroprevalence of HHV8 was relatively high compared with that in the United States and has been found to increase statistically significantly

and steadily with age (from birth, through childhood, and into adult life) and to decrease with increasing level of education. In black hospital patients in Johannesburg and Soweto, the age- and sex-standardized seroprevalence of HHV8 was slightly more than 30%, compared with 20% in black blood donors and about 5% in white blood donors, and it did not vary by sex (23). In a rural South African (black) population, seroprevalence rates were even higher (27). These findings are echoed by those from elsewhere in Africa, where HHV8 seroprevalence is also high and has been found to increase with age, suggesting that the virus is not a newly introduced sexually transmitted infection in Africa, as it may be in the United States (29). Furthermore, the lower seroprevalence of HHV8 in whites than in blacks in South Africa and the decrease in seroprevalence with increasing education (23) might suggest that factors associated with poverty contribute to transmission of the virus.

The presence of anti-HHV8 antibodies in infants suggests that transmission of HHV8 from mother to child is likely (27). A study of South African mothers and their children found that about 30% of the children (<10 years old) of HHV8-seropositive mothers were themselves HHV8 seropositive, whereas none of the children of HHV8-seronegative mothers were themselves HHV8 seropositive (28). Furthermore, the proportion of children who were seropositive for HHV8 increased in relation to their mothers' HHV8 antibody titer; although inconclusive, the data suggested that HHV8-seropositive mothers with high-titer infection may be about twice as likely to have HHV8-seropositive children as the mothers with low-titer infections (30). The steady increase in the prevalence of HHV8 infection throughout childhood suggests that transmission of the virus from person to person, via nonsexual routes, may also occur (27,29). A study in Uganda (29) showed that HHV8 seropositivity in children was strongly associated with the presence of antibodies to hepatitis B core antigen. Hepatitis B is known to be transmitted from person to person, and this may suggest a similar route for HHV8 (31).

SUMMARY AND FUTURE RESEARCH

Little doubt exists that HHV8 is responsible for most, if not all, cases of Kaposi's sarcoma. However, many questions remain unanswered about the etiology of this tumor. Why, for example, is Kaposi's sarcoma more common in men than in women in South Africa, when the prevalence of HHV8 is the same? The association between high anti-HHV8 antibody titers and risk of Kaposi's sarcoma is clear, but it is not known if those antibody titers are persistently high, prior to the diagnosis of the tumor, nor is it known what the determinants of high antibody titer are. It is assumed that anti-HHV8 antibody titers reflect the viral load of HHV8, but little evidence exists for this assumption, and, for a given HHV8 titer, the exact mechanism by which HIV has such a dramatic impact on the risk of Kaposi's sarcoma is not clear.

The association of HHV8 seropositivity with poor education and low social class in South Africa is in complete contrast to the risk factors for Kaposi's sarcoma identified in studies from Uganda (32,33). The development of Kaposi's sarcoma in HIV-seronegative and in HIV-seropositive individuals in Uganda is associated with markers of high social class, such as better education and wealth. Furthermore, despite the fact that HHV8 is very prevalent in Uganda, Kaposi's sarcoma is a relatively uncommon manifestation of HIV disease, occurring in less than 7% of cases (10). The interpretation of these findings is difficult;

however, if high social status protects an individual from early infection with HHV8, it could imply that the age at which infection occurs (or even the route of infection) affects the subsequent risk of Kaposi's sarcoma. This hypothesis is reminiscent of the effect of infection in adult life with the Epstein-Barr virus (a closely related gamma herpesvirus) in relation to the risk of infectious mononucleosis.

Kaposi's sarcoma is one outcome of infection with HHV8, both in HIV-seropositive and in HIV-seronegative adults and children. In children, it is possible that the tumor is a manifestation of primary infection with HHV8, although this is speculative; in adults, all of the available evidence suggests that Kaposi's sarcoma occurs after primary infection (17). Almost no data are available on the clinical manifestations, if any, of primary infection with HHV8; therefore, there is no understanding of how important those manifestations might be in terms of morbidity. In a case report (34), transient angiolymphoid hyperplasia was found to occur as part of an HHV8 seroconversion syndrome in an HIV-infected adult. Nothing is known about the clinical manifestations of primary HHV8 infection in HIV-seropositive or HIV-seronegative children or in HIV-seronegative adults.

In South Africa, about a third of the children of HHV8-seropositive mothers are themselves HHV8 seropositive, but the determinants of transmission from an HHV8-seropositive mother to her child are unknown. Maternal anti-HHV8 antibody titers may be important and probably reflect the number of circulating HHV8-infected cells (i.e., the viral load), although this area needs clarification. The role of other factors, such as co-infection with HIV, maternal age at delivery, mode and place of delivery, and length of breast-feeding in relation to mother-to-child transmission of HHV8, remains to be investigated. It is not even known if HHV8 is present in breast milk, although it has been identified in saliva (35). Similarly, if person-to-person transmission occurs via nonsexual routes, other than from a mother to her child, little is known about the mechanism or possible outcomes of this transmission.

Incidence rates for Kaposi's sarcoma in South Africa are rising rapidly, much as they did in Kampala at the beginning of the AIDS epidemic. Applying the age-specific incidence rate of Kaposi's sarcoma, estimated from the Kampala Cancer Registry (10), to the South African black population would lead to an additional 8000 cases of Kaposi's sarcoma in South Africa and an increase in the overall population lifetime risk (0-74 years) of developing a cancer from about 1 in 4 to 1 in 3.5 (12).

Finally, Kaposi's sarcoma is being increasingly reported in HIV-seronegative homosexual men in New York and in HIV-seronegative children in Africa (36,37). If the recent spread of HIV in the South African population has also led to an increase in the spread of HHV8 infection, this spread may result in an increase in the incidence of Kaposi's sarcoma even in people who are not infected with HIV.

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NOTES

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Immunodeficiency, Immunosuppression, and Susceptibility to Neoplasms

Robert S. Schwartz

HISTORICAL BACKGROUND

The idea that the immune system serves as a protective barrier against the growth of neoplastic cells began to emerge at the end of the 19th century, soon after the role of immunity in the defense against infection was established. In 1908, the year he was awarded the Nobel Prize, Paul Ehrlich wrote, "... in the enormously complicated course of fetal and post-fetal development, aberrant cells become unusually common. Fortunately, in the majority of people, they remain completely latent thanks to the organism's positive mechanisms" (1). These "positive mechanisms," Ehrlich proposed, were grounded in the immune system, and, when they were depressed, "the rapid, parasitic growth of [neoplastic] cells" would follow. Twenty years later, in his book *Heteroplastic and Homoplastic Transplantation*, Georg Schoen (2) coined the term "transplantation immunity" and formulated the general laws of transplantation as we know them today: Transplantation into a foreign species or unrelated members of the same species fail, whereas autografts succeed; a second graft in a recipient that previously rejected a graft from the same donor undergoes accelerated rejection; and a close "blood relationship" between donor and recipient enhances the success of the graft.

It is notable that all these principles had been demonstrated in experimental animals with the use of grafts of tumor cells. In that same era, James B. Murphy (3) called attention to the role of the lymphocyte in the rejection of tumor grafts, and George D. Snell (4), another Nobel laureate, began his historic experiments that were to become the foundation of immunogenetics. The discovery by Peter Gorer (5) of hemagglutinating antibodies in the serum of mice that had rejected a tumor allograft (containing what he called antigen II) was joined with Snell's H locus—the genetic basis of the rejection of tumor allografts—to yield the H-2 system. For all these reasons, tumor immunology became inextricably linked to transplantation immunology and, thus, back to Ehrlich's idea that one of the functions of the immune system is to guard against the growth of neoplastic cells. [The history of transplantation immunology is well summarized in (6).]

Despite these historic advances, the idea of protective immunity against autochthonous neoplasms remained in the backwater of immunology until 1970, when F. McFarlane Burnet aroused interest in the topic with his book *Immunological Surveillance* (7). In this work, Burnet presented the sweeping hypothesis that "... an important and possibly primary function of the immunological mechanisms is to eliminate cells which as a result of somatic mutation or some other inheritable change represent potential dangers to life." Burnet assigned this function to the cellular immune system (today's T cells) and went on to write, "... without immunological surveillance, cancer would be more frequent and occur at younger ages than it does," and that "immuno-suppressive agents (sic) ... will increase the likelihood of neoplasia." Thirty years after its publication, this book

remains a fascinating glimpse into the thoughts of a brilliant theorist, yet, despite his sparkling imagination, Burnet had to admit that he could not conceive of an experiment that would conclusively prove his thesis. Indeed, evidence that the immune system has a major role in defending the body against mutated (neoplastic) cells is still wanting.

NEOPLASMS IN CONGENITAL IMMUNODEFICIENCY DISEASES

One would suppose that an excellent approach to finding ways of testing Burnet's idea is through studies of congenital immunodeficiency diseases, in which the hypothetical immunologic surveillance system would be severely impaired, hence rendering the patient susceptible to cancers of all types. But the congenital immunodeficiency diseases may not be the ideal testing ground for immunologic surveillance [see Table 1 and (8)]. For example, there is no undue susceptibility to cancer in X-linked hypogammaglobulinemia because the defect is in B cells; T-cell immunity, which is thought to be crucial for immunologic surveillance, is intact (9). Severe combined immunodeficiency disease, the DiGeorge syndrome, and congenital deficiency of major histocompatibility complex molecules are usually fatal within the first year of life unless corrected by a bone marrow or thymic transplant. There is simply insufficient time for a neoplasm to develop in children with these disorders.

In the X-linked hyperimmunoglobulin M (hyper-IgM) syndrome, common variable immunodeficiency disease, and selective immunoglobulin A (IgA) deficiency, there does seem to be an increased risk of neoplasms, but these diseases are almost always lymphomas rather than a representative sample of the variety of neoplasms that affect children (10,11). Moreover, evidence directly implicating the immunodeficiency itself as the cause of the lymphomas in these three diseases is inconclusive. Patients with X-linked hyper-IgM syndrome, common variable immunodeficiency disease, or selective IgA deficiency have a disorder of immunoregulation, often manifested clinically by enlarged lymph nodes and splenomegaly. The derangement of the immune system—and not the immunodeficiency—may be the key reason for the development of lymphomas in these patients. In the X-linked hyper-IgM syndrome, for example, the defect is a crippling mutation in the CD40 gene, which encodes a protein (CD40) that is required for the production of immunoglobulin G (IgG) antibodies (12). In addition to this property, the CD40 molecule and its ligand have anti-apoptotic properties in normal and neoplastic B cells (13–17). In common variable immunodeficiency and IgA deficiency, there is not only impaired immunoregulation but also chromosomal abnormalities, both of which may contribute to the susceptibility to lymphomas (18).

Correspondence to: Robert S. Schwartz, M.D., *New England Journal of Medicine*, 10 Shattuck St., Boston, MA 02115 (e-mail:rschwartz@nejm.org).

See "Note" following "References."

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Table 1. Congenital immunodeficiency diseases and susceptibility to neoplasms

Disease	Neoplasms
X-linked hypogammaglobulinemia	No
Severe combined immunodeficiency disease	No
DiGeorge syndrome	No
Major histocompatibility complex disease	No
Hyperimmunoglobulin M syndrome	Yes
Common variable immunodeficiency	Yes
Selective immunoglobulin A deficiency	Yes
X-linked lymphoproliferative disease	Yes
Wiskott-Aldrich syndrome	Yes
Ataxia-telangiectasia	Yes
Nijmegen breakage syndrome	Yes

An increased susceptibility to lymphomas has also been noted in the Wiskott-Aldrich syndrome of thrombocytopenia, eczema, recurrent otitis media, and susceptibility to infection by a variety of microorganisms (19). The faulty WAS gene has been mapped to chromosome Xp11.22, and the Was protein, located in the cytoplasm, is probably involved in signal transduction and the maintenance of the cytoskeleton in many kinds of cells, including all three lineages of hematopoietic cells and their precursor, the CD34⁺ hematopoietic stem cell (20). Many aspects of the function of the Was protein are unknown, but it is plausible that disorganization of a protein with such fundamental properties could increase the probability of the malignant transformation of a cell. Ataxia-telangiectasia, the Nijmegen breakage syndrome, and Bloom syndrome, all associated with immunodeficiency, are really diseases of chromosomal instability because of faults in the mechanism of DNA repair. This is why patients with ataxia-telangiectasia and cultures of their cells are extremely susceptible to radiation (15). Malignancies, mainly lymphomas, occur in about one third of homozygotes for the ataxia-telangiectasia defect or the Nijmegen breakage syndrome (15). The cause of these neoplasms is likely to be the marked chromosomal instability, but the immunodeficiency may have a secondary role by allowing the early appearance and rapid growth of the tumors.

X-linked immunodeficiency disease (also called X-linked lymphoproliferative disease and Duncan's disease) is of special interest because of its association with lymphomas caused by the Epstein-Barr virus (EBV) (21). Boys who carry the XLP gene are healthy until they become infected with EBV. The outcome of this infection can be a severe, often fatal form of infectious mononucleosis, a malignant lymphoma, or hypogammaglobulinemia, sometimes with increased serum levels of IgM. Lymphoma develops in about one third of patients with X-linked lymphoproliferative disease, usually around the age of 5 or 6 years (21). Grierson and Purtilo (22) have estimated that the risk of lymphoma in boys with the disease is 200 times greater than that in the general population. The lymphomas usually appear in extranodal sites, arise from B cells, and often have the histologic features of Burkitt's lymphoma. Other types of lymphoma, including immunoblastic, large noncleaved or small cleaved, and mixed cell lymphomas, also occur in this disease (23). Before infection by EBV, boys with X-linked lymphoproliferative disease may have subtle immunologic defects, such as impaired isotype switching (IgM to IgG), but the outstanding feature after infection by the virus is the lack of anti-EBV antibodies. The XLP gene, located on chromosome Xq25, has been cloned and found to be a mutated version of a gene called SH2D1A, which encodes a protein involved in multiple intracellular signaling

pathways, especially in T cells [reviewed in (24)]. This rare disease is an experiment of nature that demonstrates the importance of the immune system in the defense against a potentially oncogenic virus. It is likely that this aspect of protective immunity is the key to understanding the development of neoplasms in recipients of allografts who receive treatment with immunosuppressive drugs.

ANIMAL MODELS

Before continuing with the main theme of this discussion, let us briefly consider the possibility that a benign lymphoproliferative disease can be the prelude to a lymphoma. Lymphoma, one of the commonest neoplasms in immunosuppressed patients, occurs through a complex, multistep process. Table 2 shows several animal models in which there is massive proliferation of lymphocytes, usually B cells. Some of these lymphoproliferative diseases arise spontaneously, as a result of a mutation in a gene that encodes immunoregulatory molecules (MRL/lpr/lpr and Gld mice); some develop in transgenic animals that carry genes related to apoptosis (Bcl-2, for example) or transcription factors (Fli-1, for example). In others, a key immunoregulatory gene is disabled (CTLA-4 or Lyn). In Bcl-2 transgenic animals (25) and CTLA-4 knockout mice (26), there is a massive proliferation of B cells (Bcl-2 transgenics) or T cells (CTLA-4 knockouts), but the proliferating B cells do not appear to be neoplastic. From these examples, we can see that an alteration in a single gene is unlikely to cause neoplastic growth of lymphocytes. The same applies to MRL/lpr/lpr and Gld mice, which carry spontaneously mutated FAS and FAS ligand genes, respectively (27). Of all the animal systems listed in Table 2, the only one in which a lymphoproliferative disease culminates in the development of a lymphoma is the chronic graft-versus-host disease model. This disorder is produced by the injection of lymphocytes from an inbred mouse (mouse "A") into an F₁ hybrid ("A × B"), using parental strains ("A" and "B") with a strong histocompatibility (H-2 antigen) difference. In the F₁ mouse, H-2 antigens from parent B constantly stimulate T cells from parent A, leading to a massive lymphoproliferative disease that culminates in a lymphoma. In this model, perhaps unlike the others, there is not only massive lymphoproliferation but also an outpouring of cytokines and other growth factors. It may be that the simultaneous pressures applied to lymphocytes by a strong antigenic stimulus and a surfeit of growth factors provide the conditions required for neoplastic transformation.

NEOPLASMS IN RECIPIENTS OF ALLOGRAFTS

From the very beginning of the clinical use of organ and bone marrow allografts, the possibility of a demanding test of the

Table 2. Development of lymphomas in animal models of lymphoproliferation

Model	Affected cell	Neoplasms
Chronic graft-versus-host disease	T + B	Yes
Bcl-2 transgenic	B	No
CTLA-4 knockout	CD4 T	No
Lyn knockout	T	No
Fli-1 transgenic	B	No
NZB	B	No
MRL/lpr/lpr	T + B	No
Gld	T + B	No

immunologic surveillance theory was at hand. All recipients of such grafts, especially organ allografts, required extended treatment with immunosuppressive drugs to prevent rejection of the graft. Recipients of marrow allografts received a rigorous preparation with total-body radiation and, to prevent graft-versus-host disease, long-term immunosuppression after receiving the allogeneic marrow. In retrospect, patients who were treated with an allograft were the harbingers of things to come because of their susceptibility to virtually all of the infectious and neoplastic complications of acquired immunodeficiency syndrome (AIDS). These complications could be traced directly to the deficiency of T cells caused by the immunosuppressive therapy.

The immunologic surveillance theory predicted an increased frequency of all the common neoplasms that affect humans because the immune systems of these patients were suppressed to the point that allowed acceptance of an allograft. That prediction was not realized, however. There was, to be sure, an increased incidence of neoplasms in this population but not an increase in the incidence of cancer of the lung, breast, or bowel. Instead, what actually occurs is a considerable increase in the frequency of cancers of the skin, B-cell lymphomas, Kaposi's sarcoma, and several unusual tumors (28–34) (Fig. 1). The risk of developing such neoplasms is related to the extent of immunosuppressive therapy (35), and, as experience with managing allograft recipients has increased, the risk of opportunistic cancer has decreased. Because recipients of allografts almost always require continuous treatment with immunosuppressive drugs, they have a life-long increased risk of neoplastic disease. For example, among patients who survived for at least 10 years with a renal allograft, the cause of death was cancer in 26% (30).

Skin cancer is among the most frequent neoplasms in recipients of organ allografts (36–38). In one study, the risk of cutaneous squamous cell carcinoma was increased 65-fold, carcinoma of the lip was increased 20-fold, and the risk of Kaposi's sarcoma was increased 84-fold, compared with the general population. The risk of these cutaneous cancers was related to the degree of immunosuppression caused by long-term immunosuppressive therapy (37). The frequency of skin cancers in the transplanted population increases with time after transplantation,

rising 40% to 70% after 10 years. The squamous cell carcinomas tend to be multiple and even life threatening (36,38).

Another category of lesions that occurs in allograft recipients is post-transplant lymphoproliferative disease. In allograft recipients, these disorders are relatively common, complex, and difficult to treat. They include polymorphic lymphoproliferative disorders, monomorphic lymphoproliferative lesions that usually arise in B cells but may also originate from T cells (39), and Hodgkin's-like lesions (40). The polymorphic lymphoproliferative disorders consist of B cells and T cells intermingled with numerous plasma cells. They are often reversible if immunosuppressive therapy is decreased or discontinued, whereas the monomorphic lesions are usually irreversible—they are monoclonal disorders with all the features of a malignant lymphoma (40–42). There is a marked tendency for the monoclonal lymphoproliferative lesions to occur in extranodal sites, especially in the central nervous system (43).

Virtually all post-transplant lymphoproliferative disorders are linked to EBV (44–46). EBV has adapted to its host by using multiple, complex, and, some might say, ingenious tactics. After infecting a resting B cell, the virus drives the cell to proliferate. At this stage, the B cell expresses multiple EBV antigens, which provide ample targets for cytotoxic T cells. Normally, these T cells eliminate the proliferating clones of EBV-infected B cells, but in some B cells the virus escapes notice by entering a latent phase in which only the LMP2a antigen is expressed. LMP2a has the capacity to mask the expression of class I HLA antigens, through which cytotoxic T cells come into contact with virus-infected cells. In a normal adult, there are about 10⁶ non-dividing, EBV-infected memory B cells; in patients receiving immunosuppressive therapy, there is a dramatic rise in such cells. Occasionally, these B cells carrying latent EBV fortuitously become activated when they come into contact with activated T cells in germinal centers; on activation, they express only the EBNA-1 antigen, which allows the virus to replicate. The EBNA protein interferes with the ability of HLA class I molecules to transport EBNA peptides to the cell surface, so these cells, too, escape immune surveillance. Ultimately, these B cells return to the resting stage and again express only LMP2a [reviewed in (47)].

It is important to note that EBV is not the sole element in the pathogenesis of post-transplant lymphoproliferative diseases. In Burkitt's lymphoma, for example, there is a genetic change in the cell, the c-myc translocation, which is essential for the tumor to evolve. In one study of 57 post-transplant lymphoproliferative disorders, mutations of the BCL-6 proto-oncogene were found in 43% of the polymorphic lesions and in 90% of the post-transplant lymphomas (48). Mutations of BCL-6 have also been found in lymphomas that were not associated with immunosuppressive therapy (49) and may play a key role in lymphomagenesis.

There is evidence that viral

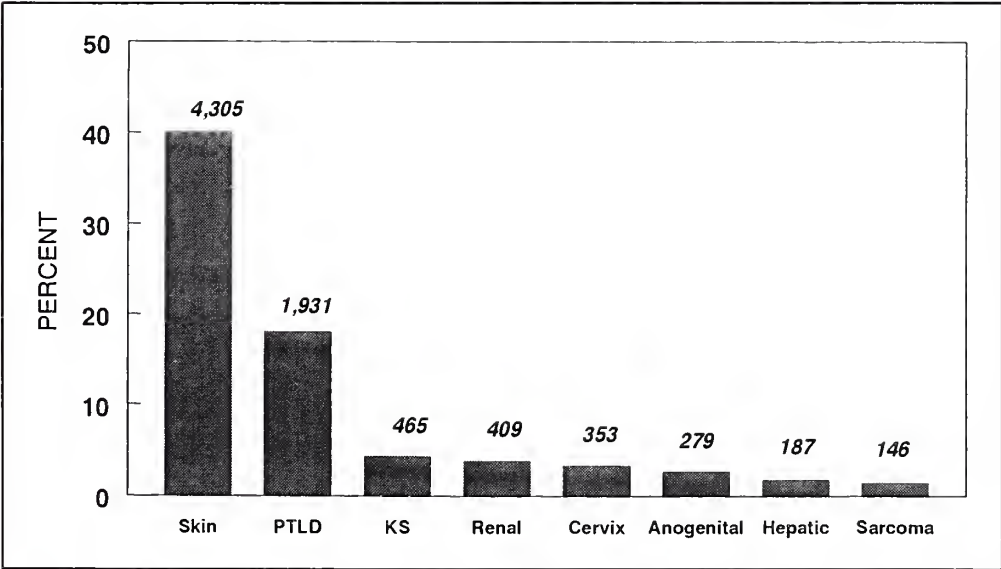


Fig. 1. Types of cancers reported in recipients of allografts (10 787 recipients; 11 483 neoplasms). PTLD = post-transplant lymphoproliferative disease; KS = Kaposi's sarcoma. [Data from (28).]

load is an important factor in susceptibility to post-transplant lymphoproliferative diseases (46,50). Moreover, Burkitt's lymphoma is monoclonal, monomorphic, and irreversible at the outset, whereas the lymphoproliferative lesions in allograft recipients are initially reversible [reviewed in (51)]. Presumably, the conversion from reversible polyclonal post-transplant lymphoproliferative disease to an irreversible monoclonal lymphoma occurs when an EBV-infected B-cell (or T-cell) clone acquires a genetic lesion that gives that clone a growth advantage over other clones in the mass of cells that proliferate without hindrance in the immunosuppressed allograft recipient.

Post-transplant lymphoproliferative disease and the lymphomas in AIDS patients have similarities and differences. Both are characterized by extranodal disease and aggressive behavior, but the post-transplant lymphoproliferative disorders are uniformly infected by EBV, whereas EBV is detectable in only about half of the lymphomas in patients with AIDS (52,53). Another difference is that Burkitt-type lymphoma is relatively common in patients with AIDS but rare in allograft recipients (54). Another interesting aspect of post-transplant lymphomas is that follicular lymphoma, the commonest lymphoma among adults, is not seen in immunosuppressed patients. The reasons for these differences are unknown, but they likely depend on the immunologic status of the patient, the presence or absence of EBV, and the types of genetic changes in the tumors.

In recipients of allogeneic bone marrow, EBV-induced lymphoproliferative disease arises in B cells from the donor (55), and the risk of such a disease developing in the recipient is reduced by depleting the allogeneic marrow of B cells (55,56). Persuasive evidence that the lack of anti-EBV immunity is an essential feature of post-transplant lymphoproliferative diseases comes from experiments in which transfusions of leukocytes (not plasma) from EBV-positive donors caused regressions of the lymphoproliferative lesions (57,58).

It is likely that the susceptibility of allograft recipients to Kaposi's sarcoma is also related to the lack of a defense against a herpesvirus—in this case, human herpesvirus 8 (HHV8). In one series of 28 cases of Kaposi's sarcoma in transplant recipients, HHV8 was found in 27 of 28 lesions by means of the polymerase chain reaction. It is possible that latent HHV8 infection in allograft recipients becomes activated after transplantation, because titers of anti-HHV8 antibodies rise and HHV8 DNA can be found in the blood after institution of immunosuppressive therapy (59,60). Regamey et al. (61) have reported that in some cases HHV8 may be transmitted from the donor to the recipient through a renal allograft.

CONCLUSION

The advent of clinical transplantation presented a major challenge to the immunologic surveillance theory. It would appear that, in the context of transplantation, the theory is wanting because recipients of allografts are not unduly susceptible to the most common cancers in humans: lung, breast, prostate, intestinal, or ovarian cancer. Instead, there is a many-fold increased risk of the development of virus-induced neoplasms because of the inability of the recipient to suppress activation of latent herpesviruses or a new infection with these agents. Nevertheless, questions remain, and it would be premature to write off the theory. For example, skin cancers are among the commonest malignant growths in allograft recipients, yet none of these neo-

plasms has yet been linked to an oncogenic virus. It is plausible that skin cells in which DNA is damaged by UV radiation occur frequently in normal people, who, by an immune response, eliminate such potentially malignant cells. In the allograft recipient, this response may be lacking, thereby allowing the growth of neoplastic cutaneous cells. Another factor deserving consideration is the possibility that the immunosuppressive drugs themselves are oncogenic. Recently, Hojo et al. (62) found that cyclosporine induced phenotypic changes in cultured normal cells that allowed them to invade normal tissues when transplanted *in vivo*. Moreover, cyclosporine caused marked alterations associated with high-grade malignancy in cultured adenocarcinoma cells. These effects were nullified by adding monoclonal antibodies against transforming growth factor- β . Investigations of allograft recipients have been a rich source of knowledge about the immune system, microbiology, oncology, and genetics, but it is apparent that we still have much to learn from these remarkable patients.

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NOTE

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Regulation of Neoplastic Angiogenesis

Isaiah J. Fidler

The progressive growth of neoplasms and the production of metastasis depend on the development of adequate vasculature, i.e., angiogenesis. The extent of angiogenesis is determined by the balance between positive- and negative-regulating molecules that are released by tumor and host cells in the microenvironment. The growth of many neoplasms is associated with the absence of the endogenous inhibitor of angiogenesis, interferon beta (IFN β). A survey of multiple mouse and human tumors shows a lack of IFN β associated with extensive angiogenesis. Therapy with IFN α or β either by subcutaneous injection of the protein or by introduction of viral vectors that contain the IFN β gene inhibit angiogenesis and, hence, progressive tumor growth. [J Natl Cancer Inst Monogr 2000;28:10-4]

CANCER METASTASIS

The major cause of death from cancer is metastases that are resistant to conventional therapy. One major obstacle to the treatment of metastasis is the biologic heterogeneity of neoplasms (1). A second obstacle is the ability of different organ environments to modify a metastatic tumor cell's response to therapy (2,3). A better understanding of the mechanisms that regulate the process by which tumor cells invade local tissues and spread to distant organs should lead to the design of more effective therapy.

The process of cancer metastasis consists of a series of sequential steps, each of which can be rate limiting (1). After the initial transforming event, growth of neoplastic cells must be progressive. Extensive vascularization must occur if a tumor mass is to exceed 1 mm in diameter (4). The next step is local invasion of the host stroma that occurs by several mechanisms (5). Small tumor cell aggregates then detach and embolize next and some tumor cells that survive the trauma of the circulatory system arrest in the capillary beds of organs extravasate into the organ parenchyma, proliferate, and induce angiogenesis to allow expansion of the lesion (1).

The outcome of metastasis depends on the interactions of tumor cells with various host factors (1,6,7). The pattern of metastasis is not random but rather is determined by factors that are independent of vascular anatomy, rate of blood flow, and the number of tumor cells delivered to each organ (1). The search for factors that regulate metastasis began in 1889 when Paget analyzed postmortem data of women who died of cancer and noticed the high frequency of metastasis to the ovaries and the different incidence of skeletal metastases associated with different primary tumors. Paget concluded that the organ distribution of metastases is not a matter of chance and suggested that metastases develop only when the "seed" (certain tumor cells with metastatic ability) and the "soil" (colonized organs providing growth advantage to the seeds) are compatible (8). In recent years, Paget's hypothesis has received considerable experimental and clinical support (1,9-11). Site-specific metastasis has been demonstrated with many transplantable tumors and has

also been documented in autochthonous human tumors in patients with peritoneovenous shunts (12,13).

A current definition of the "seed and soil" hypothesis encompasses three principles. First, neoplasms are biologically heterogeneous (1,14). Second, the process of metastasis is highly selective, favoring the survival and growth of a small subpopulation of cells that pre-exist in the heterogeneous parent neoplasm (6). Third, the outcome of metastasis depends on multiple interactions of metastatic cells (seed) with homeostatic mechanisms (soil) (2). The majority of malignant neoplasms actually usurp homeostatic mechanisms to gain growth advantage (1,6,7). Neoplastic angiogenesis is an excellent example.

TUMOR ANGIOGENESIS

The survival and growth of cells depend on an adequate supply of oxygen and nutrients and on the removal of toxic molecules. Oxygen can diffuse from capillaries for only 150-200 μ m. When distances of cells from a blood supply exceed this, cell death follows (15). Thus, the expansion of tumor masses beyond 1 mm in diameter depends on neovascularization, i.e., angiogenesis (4,16). The formation of new vasculature consists of multiple, interdependent steps. It begins with local degradation of the basement membrane surrounding capillaries, followed by invasion of the surrounding stroma and migration of endothelial cells in the direction of the angiogenic stimulus. Proliferation of endothelial cells occurs at the leading edge of the migrating column and the endothelial cells begin to organize into three-dimensional structures to form new capillary tubes (4,17). Differences in cellular composition, vascular permeability, blood vessel stability, and growth regulation distinguish vessels in neoplasms from those in normal tissue (18).

The onset of angiogenesis involves a change in the local equilibrium between proangiogenic and antiangiogenic molecules (19). The major proangiogenic molecules include fibroblast growth factor (FGF) family members, vascular endothelial cell growth factor or vascular permeability factor (VEGF/VPF), interleukin 8 (IL-8), angiogenin, platelet-derived endothelial cell growth factor, platelet-derived growth factor, and matrix metalloproteinases (4,20,21). Many different proangiogenic or antiangiogenic molecules are present in different tissues (4,22). In normal tissues, factors that inhibit angiogenesis predominate (e.g., interferon beta [IFN β], tissue inhibitor of metalloproteinases) (4,23), whereas, in rapidly dividing tissues, factors that stimulate angiogenesis predominate. Our laboratory has investigated the role of cell density in the regulation of bFGF expression in human renal cell carcinoma cells or human endothelial cells. Dividing cells expressed higher levels of bFGF (both at

Correspondence to: Isaiah J. Fidler, D.V.M., Ph.D., Department of Cancer Biology, Box 173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 (e-mail: ifidler@notes.mdacc.tmc.edu).

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messenger RNA [mRNA] and protein levels) than nondividing cells (24). In contrast, nondividing cells express higher levels of VEGF/VPF than dividing cells (25).

REGULATION OF ANGIOGENESIS BY THE MICROENVIRONMENT

The production of bFGF and IL-8 by tumor or host cells or the release of angiogenic molecules from the extracellular matrix induces the growth of endothelial cells and the formation of blood vessels. Data from our laboratory have demonstrated that the organ microenvironment can directly contribute to the induction and maintenance of the angiogenic factors bFGF (26,27) and IL-8 (28). For example, in patients with renal cell carcinoma, the level of bFGF in the serum or urine inversely associated with survival (29,30). Human renal cancer cells implanted into different organs of nude mice had different metastatic potentials: Those implanted into the kidney produced a high incidence of lung metastasis, whereas those implanted subcutaneously were not metastatic (26). Histopathologic examination of the tumors revealed that subcutaneous tumors had few blood vessels, whereas the tumors in the kidney had many (26). The subcutaneous (or intramuscular) tumors had a lower level of mRNA transcripts for bFGF than did continuously cultured cells, whereas tumors in the kidney of nude mice had 20-fold the levels of bFGF mRNA and protein level (26,27).

Constitutive expression of IL-8 directly associates with the metastatic potential of the human melanoma cells (28). IL-8 contributes to angiogenesis by inducing proliferation, migration, and invasion of endothelial cells (31). Several organ-derived cytokines (produced by inflammatory cells) can increase expression of IL-8 in normal and tumorigenic cells (32). IL-8 expression was increased in co-culture of melanoma cells with keratinocytes (skin), whereas it was inhibited in cells co-cultured with hepatocytes (liver). Similar results obtained with conditioned media from keratinocyte and hepatocyte cultures suggested that organ-derived factors, e.g., IL-1 and transforming growth factor- β , can modulate the expression of IL-8 in human melanoma cells (32).

The influence of the microenvironment on the expression of VEGF/VPF, angiogenesis, tumor cell proliferation, and metastasis was investigated with the use of human gastric cancer cells implanted in orthotopic (stomach) and ectopic (subcutaneous) sites in nude mice. Tumors in the stomach were highly vascularized and expressed higher levels of VEGF/VPF than did subcutaneous tumors (33). Moreover, only tumors implanted in the stomach produced metastasis, suggesting that the expression of VEGF/VPF vascularization and metastasis of human gastric cancer cells are regulated by the organ microenvironment.

MOLECULAR DETERMINANTS OF ANGIOGENESIS IN CUTANEOUS HEMANGIOMAS

Infantile cutaneous hemangiomas represent a unique form of pathologic angiogenesis in which endothelial cell tumors grow rapidly in the first year of life (proliferative phase), followed by a slow regression during the next 5 years (involuting phase) and eventual involution or complete regression (involved phase) by the age of 10–15 years (34). Long-term daily systemic treatment with IFN α has been shown to accelerate the involution of fatal hemangiomas (34–39). To determine whether the progression and involution of infantile cutaneous hemangiomas were asso-

ciated with overexpression of proangiogenic molecules or the lack of antiangiogenic molecules, a large number of hemangioma specimens by immunohistochemistry was analyzed. The results showed that proliferating hemangiomas expressed bFGF and VEGF/VPF but not IFN β (mRNA and protein) (40). A surprising finding was that the epidermis directly overlying proliferating hemangiomas was hyperplastic, whereas the epidermis overlying involuted hemangiomas or the epidermis from an unaffected site was not (40). The hyperplastic epidermis expressed bFGF, VEGF/VPF, and IL-8 but not IFN β , whereas the normal epidermis expressed both positive- and negative-angiogenic molecules (40). These data raised the possibility that the proliferating hemangiomas induced hyperplasia in the surrounding normal tissues (epidermis), leading to production of bFGF and VEGF/VPF but not IFN β (40), supporting the concept that neoplastic cells subvert and usurp host homeostatic mechanisms for their growth advantage (1,2).

To study the relationship between hemangiomas and the microenvironment, an *in vivo* model was developed for epidermal hyperplasia and angiogenesis, using UVB irradiation of mice (41). Mice exposed to 10 kJ/m² UVB developed epidermal hyperplasia accompanied by angiogenesis and telangiectasia during the first week after irradiation, but these conditions slowly subsided over the following weeks. The first striking event after UVB irradiation was the increase in production of bFGF in the keratinocytes of the epidermis (41). The increase in bFGF preceded or at least coincided with the division of epidermal cells recognized by immunohistochemical staining with antibodies to proliferating cell nuclear antigen. Marked hyperplasia and angiogenesis followed immediately. The expression of VEGF/VPF was slightly increased by day 5. Of interest, the expression of IFN β in the epithelium decreased with epidermal hyperplasia but was re-expressed as the hyperplasia and angiogenesis subsided (42).

Systemic therapy with the use of recombinant IFNs produces antiangiogenic effects in vascular tumors, including hemangioma (34–39), Kaposi's sarcoma (43–46), melanoma (47), basal cell and squamous cell carcinomas (48), and bladder carcinoma (49). These tumors have also been documented as producing the high levels of bFGF often detectable in the urine or serum of these patients (29,30,50). These findings, along with our *in vivo* observations, prompted us to investigate whether IFNs could modulate the expression of the angiogenic molecule bFGF. We found that IFN α and IFN β but not IFN γ decreased the expression of bFGF mRNA and protein in human renal cell cancer (HRCC) as well as in human bladder, prostate, colon, and breast carcinoma cells (51). The inhibitory effect of IFN α and β on bFGF expression was cell-density dependent and independent of the antiproliferative effects of IFNs (51,52). We also confirmed that IFN can inhibit bFGF production in an *in vivo* model system. Systemic administration of human IFN α decreased the *in vivo* expression of bFGF, decreased blood vessel density, and inhibited tumor growth of a human bladder carcinoma implanted orthotopically in nude mice (53).

ANTIANGIOGENIC ACTIVITY OF IFN β

The IFN family consists of three major glycoproteins that exhibit species specificity: leukocyte-derived IFN α , fibroblast-derived IFN β , and immune cell-produced IFN γ . Although IFN α and IFN β share a common receptor (the type I IFN receptor) and induce a similar pattern of cellular responses, cer-

tam cellular reactions can be stimulated only by IFN β , probably by the phosphorylation of a receptor-associated protein that is uniquely responsive to IFN β (54). In addition to their well-recognized activity as antiviral agents, IFNs regulate multiple biologic activities, such as cell growth (55,56), differentiation (57), oncogene expression (58,59), host immunity (60–62), and tumorigenicity (63–68). IFNs can also inhibit a number of steps in the angiogenic process. IFN has antiproliferative properties, especially on tumor cells (69–71), an effect that has also been demonstrated on endothelial cells *in vitro*. IFN α can inhibit FGF-induced endothelial proliferation (72), and IFN γ can inhibit endothelial proliferation (73). IFN α and IFN γ have been shown to be cytostatic to human dermal microvascular endothelial cells (74) and to human capillary endothelial cells (75).

The antiangiogenic effect of IFNs cannot be explained solely on the basis of inhibition of endothelial cell proliferation. For example, IFN α/β can also inhibit the endothelial cell migration step of angiogenesis (76,77). Subcutaneous injection of IFN α/β adjacent to a wound delayed the healing process by inhibiting the proliferation, migration, and invasion of capillary buds, fibroblasts, and epithelium (78,79). IFN α/β injected intratumorally or peritumorally into tumor cells resistant to the antiproliferative effects of IFN damages blood vessels, leading to ischemia and necrosis (80). Moreover, we reported that IFN α/β can affect the expression of several angiogenic factors, including bFGF (52,53), IL-8 (81), and collagenase type IV (82,83).

Our laboratory recently demonstrated that IFN β gene therapy can eradicate tumor cells of various histologic origins and found that the sustained local production of murine IFN β could inhibit the tumorigenicity and metastasis of human and murine tumor cells implanted into nude mice (84,85). All human tumor cell lines transfected with the murine IFN β gene grew well *in vitro*, but none grew *in vivo*. IFN β -transfected cells prevented the outgrowth of parental or control-transfected cells when injected at the same site but not when injected at distant sites, suggesting that IFN β promoted a local lysis of the bystander cells (84,85). Similar results were found when human prostate cancer cells were infected with the murine IFN β gene with the use of a retroviral vector. Of interest, the transduced cells did not grow in nude mice when injected into the prostate. The regression of the tumors was directly associated with infiltration by macrophages and activation of inducible nitric oxide synthase (86). All transfected and transduced cells stimulated a high level of nitric oxide in murine macrophages, which associated with the vigorous antitumor activities. Therefore, the local production of IFN β can suppress tumorigenicity and metastasis, in part because of the activation of host effector mechanisms.

CONCLUSIONS

The angiogenesis within and surrounding neoplasms is due to an imbalance between proangiogenic molecules, e.g., bFGF, VEGF/VPF, IL-8, and antiangiogenic molecules (e.g., IFN). Tumor cells, normal host cells, and leukocytes all contribute to angiogenesis. The absence of IFN β from tumor beds is associated with robust angiogenesis. Restoring the balance between proangiogenic and antiangiogenic molecules provides an approach to the control of angiogenesis in neoplasms. Frequent systemic administrations of low-dose IFN α or β or the introduction of the IFN β gene to the tumor bed show great therapeutic promise in several animal models. Clinical trials should

determine whether this approach is useful for therapy of human neoplasms.

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NOTES

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Human Herpesvirus 8 K1-Associated Nuclear Factor-kappa B-Dependent Promoter Activity: Role in Kaposi's Sarcoma Inflammation?

Felipe Samaniego, Shibani Pati, Judith E. Karp, Om Prakash, Debashish Bose

Background: The growing number of human immunodeficiency virus type 1 (HIV-1) infections worldwide and the increasing use of immunosuppressive modalities for organ transplantation have contributed to an epidemic of Kaposi's sarcoma (KS), which has been etiologically linked to human herpesvirus 8 (HHV8) or KS-associated virus. Since the onset of the acquired immunodeficiency syndrome epidemic, inflammation has been recognized as an essential component of KS pathology. HHV8 bears a gene (K1) encoding a transmembrane protein with an immunoreceptor tyrosine-based activation motif. This motif is present in receptors that mediate inflammation. **Purpose:** To dissect the cellular effects of K1 function and the eventual role of K1 in KS, we developed a cell model for studying K1 expression. **Methods:** K1 was cloned from BC-3 lymphoma cells. To monitor transcriptional activation, K1 was coexpressed with plasmids containing luciferase under control of various promoters. K1 expression was monitored by indirect immunofluorescence and by combined immunoprecipitation/immunoblot analysis. Inflammatory cytokines were measured by enzyme-linked immunosorbent assay. **Results:** Cellular transfection of the K1 gene induced reporter expression under control of nuclear factor-kappa B (NF- κ B), which controls the transcription of numerous proteins involved in inflammation. Treatment of cells with aspirin, an agent that targets this intracellular pathway and blocks cell inflammatory responses, blocked K1-induced NF- κ B-dependent promoter activity. When a second KS cofactor, i.e., the HIV-1-transactivating gene tat, was coexpressed with K1, we observed an additive effect on NF- κ B-dependent transcription. K1 transfection stimulated the secretion of cytokines interleukin (IL) 6, granulocyte-macrophage colony-stimulating factor, and IL-12. Cells treated with the conditioned media of K1 transfectants exhibited similar characteristics of K1 transfectants, indicating that a paracrine loop was being activated. **Conclusion:** Thus, K1 may activate cells in which it is expressed, as well as other cells in a paracrine manner. K1 cooperates in signaling with HIV-1 Tat, suggesting that both of the proteins from these viruses converge to reach an enhanced level of inflammation that may underlie progressive KS. [J Natl Cancer Inst Monogr 2000;28:15-23]

Kaposi's sarcoma (KS) can be a lethal disorder that preferentially occurs in clinical settings of altered immunity, as is found in human immunodeficiency virus type 1 (HIV-1)-infected individuals and in recipients of solid-organ transplants. The growing number of cases of HIV-1 infection worldwide and the increasing use of immunosuppressive drugs for organ transplantation are accompanied by a concomitant increase in cases of KS. Human herpesvirus 8 (HHV8) has been etiologically

implicated in KS, and the presence of HIV-1 further increases the rates of KS by more than 20000-fold (1-5). HIV-1 infection appears to contribute directly to KS because other retroviruses (i.e., human T-cell leukemia/lymphoma virus) that also induce immunosuppression do not predispose individuals to develop KS. In addition, other contributing factors have yet to be identified because the vast majority of the world's population of HHV8-infected persons, including those from geographic regions with high (>50%) seroprevalence, do not develop KS (6-8).

HHV8 is a recently isolated gamma herpesvirus that is related to the tumorigenic viruses herpesvirus saimiri and Epstein-Barr virus. These viruses are associated with tumor induction, particularly during immunosuppression. Their genomes contain numerous human oncogene homologues, such as cyclin D, immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling proteins (*see below*), bcl-2, and cytokine homologues of monocyte inflammatory protein 1 (MIP-I) and MIP-II, thereby making them resourceful models for comparative studies of viral oncogenesis (9,10).

The unique hyperplastic features of KS lesions have led investigators to conclude that these lesions are distinctly different from tumors of neoplastic cells. The early stages of KS are characterized by the presence of activated endothelial cells (ECs), inflammatory cell infiltration, spindle-shaped cells of vascular origin, and angiogenesis (11,12). The spindle cell population is dominated by activated ECs and macrophages (13,14) that over time proliferate to become the predominant cell phenotype.

We have shown that inflammation is a feature of KS and that inflammatory cytokines are necessary for maintaining the spindle cell phenotype found in these lesions. ECs and monocytes are considered to be the progenitors of KS spindle cells because they acquire the spindle cell morphology, marker expression, and functional features of KS spindle cells when exposed to inflammatory cytokines (tumor necrosis factor- α , interleukin [IL] 1 β , and interferon gamma) (15-17). Inflammatory cytokines induce ECs to produce (30-fold) more basic fibroblast growth factor and vascular endothelial growth factor that are essential for generating the angiogenic features of KS lesions (15,16,18,19). Inflammatory cytokines also render primary ECs with a capacity to induce angiogenic KS-like lesions when im-

Affiliations of authors: F. Samaniego, Departments of Lymphoma/Myeloma and Clinical Cancer Prevention, The University of Texas M. D. Anderson Cancer Center, Houston; S. Pati, D. Bose (Institute of Human Virology), J. E. Karp (The Greenebaum Cancer Center), University of Maryland, Baltimore; O. Prakash, Alton Ochsner Medical Foundation, New Orleans, LA.

Correspondence to: Felipe Samaniego, M.D., Department of Lymphoma/Myeloma and Clinical Cancer Prevention, The University of Texas M. D. Anderson Cancer Center, Box 429, 1515 Holcombe Blvd., Houston, TX 77030 (e-mail fsamaniego@mdanderson.org).

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planted in nude mice. Moreover, inflammatory cytokines also increase the level of HHV8 DNA in blood-derived cells (11,20). Taken together, inflammation plays a key role in the development of many of the features of KS, and it is possible that the local source for inflammation may stem from the expression of the HHV8 K1 gene (*see below*).

HIV-1 contributes directly to KS development through production of HIV-1 Tat (21–23). Tat is essential for viral gene expression, yet it also exhibits the capacity to exit live cells, disseminates systemically, and enters other cells where it can activate latent HIV-1 and promoters of other genes (i.e., transforming growth factor- β and tumor necrosis factor- α) (24). Mice made transgenic for tat express the protein in blood and show preferential KS tumor growth when inoculated with KS cells (21,25,26). Thus, Tat is a systemically distributed viral cytokine that can participate in inflammation and tumor promotion.

Recipients of solid-organ transplants, especially renal transplant patients, are at increased risk of developing KS. It is believed that immune stimulation from engrafted allogeneic tissue and challenges by microbes in the setting of drug-induced immunosuppression provide the necessary conditions that would promote tissue inflammation.

Multiple studies, including our current data, suggest that HHV8 infection may play a role in host cell activation and proliferation to produce KS lesions. Even though a minority (approximately 5%) of KS spindle cells express HHV8 lytic-phase genes, expression of selected HHV8 genes may influence host cells sufficiently to induce diffusible inflammatory cytokines in a paracrine manner. It is interesting that EC proliferation with HHV8 infection has been demonstrated by Flore et al. (27). Despite the fact that only 5% of the ECs were infected with HHV8, all cells, including uninfected cells, exhibited an extended replicative life span beyond senescence with acquired telomerase activity, thereby indicating a potent paracrine effect of HHV8 (27). Also, HHV8 and ECs inoculated under the human skin of a human skin–severe combined immunodeficiency (SCID) mouse chimera produced angiogenic lesions similar to human KS (28).

Among HHV8 genes, K1 is a promising candidate for mediating activation signal pathways based on its characteristic cytoplasmic motif (29,30). K1 contains a cytoplasmic ITAM (31). ITAMs are contained in subunits of multiprotein complexes, B-cell receptor, and T-cell receptor that are critically involved in inflammatory responses (32). Thus, we anticipate K1 to signal in and activate inflammation-related pathways by mimicking the functions of host ITAM proteins (33).

The ITAM of K1 has been shown to transmit signals when this motif is tested as a chimeric protein fused to the extracellular domain of CD8 (31). This construct, however, may not reliably reflect native K1 function because the vast majority of this fused protein is CD8. ITAMs can also exhibit paradoxical effects that clearly depend on the position on the polypeptide. One isolate of K1 (clade 3A) has been shown to stimulate nuclear factor of activated T-cell (NFAT)-dependent promoter activity (30). Because ITAM-dependent signaling typically stimulates several types of promoters and because multiple isolates of K1 exist, we sought to examine 1) whether other isolates of K1 are active in promoter activity, 2) whether K1 expression would stimulate promoters of other types, 3) whether K1 is expressed in KS tumor and cell lines, and 4) whether K1 cooperates with other factors in promoting inflammation of KS.

METHODS

Cloning

The open reading frame K1 of HHV8 was cloned by polymerase chain reaction (PCR) from DNA of BC-3 cells (provided by E. Cesarman, Cornell Medical College, New York, NY) (34,35). Thirty-five cycles (95 °C for 30 seconds, 58 °C for 60 seconds, and 72 °C for 90 seconds) were performed by PCR (1) with the use of the AmpliTaq Gold PCR Kit (The Perkin-Elmer Corp., Foster City, CA), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, and 0.001% (wt/vol) gelatin and oligomers (5'-GACGGATCCAGACCTTGTGGACATCCTG-3' and 5'-TTTTATGTAAAATACTCCAGCCCTAGGGTG-3'). Gel electrophoresis was performed, and a single prominent band of separated PCR products of approximately 1 kilobase (kb) was extracted from the gel and cloned into pCR2.1 with the use of TA cloning (Invitrogen Corp., Carlsbad, CA). The insert was sequenced. The *Bam*HI fragment containing K1 was cloned into pSG5 and pCR3.1. To epitope tag K1, we generated a fusion construct in pCR3.1 by PCR with the use of pCR2.1K1 and with oligomers 5'-CACAAGCTTCGCGAATTCATGTTCTTG-TATGTTGTTTGCAGTCTGG-3' and 5'-GATATCCCCG-GATCCCTACAGATCTTCTTCAGAAATAAGTTTTTGT-TCGTACCAATCCACTGGTTGCGTA-3' that directed the addition of the DNA coding for c-myc epitope that is recognized by monoclonal antibody 9E10: EQKLISEEDL. Constructs were sequenced to confirm DNA sequence.

Cells and Transfection

KS-1 and BC-3 cells are HHV8-infected lymphoma cells derived from primary effusion lymphoma (provided by H. P. Koefler, University of California at Los Angeles, and E. Cesarman) (36,37). They were propagated in RPMI-1640 medium with 15% fetal bovine serum and supplemented with 1 mM glutamine, penicillin G (100 U/mL), and streptomycin (100 mg/mL). Cos-1 (American Type Culture Collection, Manassas, VA) cells were selected for studying K1 expression because of their ease of transfection and their support of high-level plasmid-driven expression. KS Y-1 cells are transformed KS-derived cells isolated from an HIV-1-infected individual with KS (38). KS Y-1 cells exhibit characteristics of KS spindle cells and, like other KS-derived cell lines, lack HHV8 DNA. Cos-1 cells and KS Y-1 cells were transfected (4×10^5) with the use of the Fugene Transfection Reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN), and, after 48 hours, 20 μ g of cell extract was mixed with the luciferase assay reagent (Promega Corp., Madison, WI) and light emission was measured over a 15-second period on a luminometer (Turner Designs, Sunnyvale, CA). Because cell stress and various stimuli (e.g., oxidation stress and high serum levels) may contribute to a nuclear factor-kappa B (NF- κ B) transcriptional response, we optimized the assay conditions by minimizing nonspecific procedure-related cell activation (in RPMI-1640 medium with 0.5% fetal bovine serum) and the minimal amount of plasmid DNA (1–2 μ g) to elicit a nuclear transcription response. In other studies, transgene expression was accomplished by mixing 10^7 cells with a total of 30 μ g of total plasmid and electroporation of cells (0.28 V, 25 μ F) on the Bio-Rad Electroporator (Bio-Rad Laboratories, Hercules, CA). Assays were done in triplicate, and the means were reported. A second plasmid, using simian virus 40 promoter to direct expression of green fluorescent protein (pGreenLantern; Strata-

gene, La Jolla, CA), was used to compare transfection efficiencies. Reporter plasmids used in these studies were pAP-1-Luc and pNF κ B-Luc (Stratagene). Test plasmids pCR3.1K1, pCR3.1K1myc, and pSG5K1myc contained K1 from BC-3 cells. Control plasmid pFC-MEKK, which directs expression of extracellular-regulated kinase kinase (MEKK), was used as a potent intracellular stimulus for NF- κ B activation (Stratagene). To begin to define the pathway K1 uses in activation, we determined whether NF- κ B transcriptional activity was sensitive to the blocking effects of aspirin (1 mM) or cyclosporin (1 μ M) that are known to abrogate NF- κ B signaling (39). After transfection, cells were refed media with or without each inhibitor and incubated for another 24 hours. Whole-cell extracts were made, and 20 μ g was added to the luciferase reagent and emissions were assayed on luminometer. Cells transfected with vector alone and cells transfected with pFC-MEKK served as negative and positive controls, respectively.

Immunofluorescence Staining and Cytokine Levels

Immediately after transfection with pSG5K1myc or pSG5, cells were seeded on 12-well slides (Erie Scientific, Portsmouth, NH) and incubated in RPMI-1640 medium with 10% fetal bovine serum. The cells were washed, air-dried, fixed with acetone at -20°C , and stained by indirect immunofluorescence. The slides were dried and permeabilized with phosphate-buffered saline, 1% bovine serum albumin, 0.1% Tween, and 5% wt/vol sucrose. The cells were treated with primary antibody 9E10 (1:100) and secondary antibody anti-mouse fluorescein isothiocyanate-conjugated antibody (1:50) (mixed with Evans blue) each for 0.5 hour at 37°C . The cells were rehydrated, and anti-fade solution (Molecular Probes, Inc., Eugene, OR) was applied before sealing with coverslips. Representative cell fields were captured on 1600 film. Cells transfected with plasmid pSVK3- β catenin-myc were used as a positive control (D. Sussman, University of Maryland). After transfection, cells were refed, and the medium was collected after 24 hours. The supernatants were handled with the use of plastic ware precoated with 0.1% bovine serum albumin in phosphate-buffered saline to avoid adherence of cytokines to surfaces. Supernatants were frozen at -80°C before measurement by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Reverse Transcription (RT)-PCR and Northern Blot Analysis

Tissue from human KS was obtained after diagnostic evaluation was completed and after consent was signed, according to the policy of our Institutional Review Board. Tissue samples were snap-frozen in a dry ice/methanol bath, and total RNA was isolated with the use of Trizol according to the instructions of the manufacturer (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Cultured cell RNA was isolated with the use of the Trizol reagent. RT was performed with the Titan RT-PCR Kit (Boehringer Mannheim Biochemicals) with the use of avian myeloblastosis virus, RT, a Taq/Pwo DNA polymerase blend, and 5'-TTTGTGCCCTAGAGTGAGTT-3' and 5'-TGACTGTGTTTGATGGTTGT-3'. For northern blots, 15 μ g of total-cell RNA was separated in 0.8% formaldehyde gel and transferred to a nylon membrane. The K1 DNA sequence was labeled by the random primer method and was used for northern blot hybridization at 42°C , and washing was done at 45°C in $1 \times$ standard saline citrate/0.1% sodium dodecyl sulfate (SDS).

Immunoprecipitation/Immunoblot Analysis

Cos-1 cells were electroporated with pSG5K1myc or with pSG5. After 48 hours, cell extracts were made in 1% Nonidet buffer (40). Immunoprecipitation was conducted with anti-myc antibody (1 μ g) and rabbit anti-mouse antibody (Santa Cruz Biotech, Santa Cruz, CA, and ICN, Costa Mesa, CA), respectively, and protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) and tumbled for 12 hours at 4°C . Immunoprecipitants were size-separated on an 8% SDS polyacrylamide gel and transferred to nitrocellulose. Blotting was carried out with anti-myc antibody (1 μ g/mL), and a duplicate filter was blotted with antiphosphotyrosine (4G10) (0.5 μ g/mL) (Upstate Biotechnology, Lake Placid, NY), and the signal was read with the use of chemiluminescence (Amersham Pharmacia Biotech).

Statistical Analysis

Where appropriate, the means are shown with standard deviation. Student's *t* test was applied to estimate the statistical significance of the mean differences (41). All *P* values are two-sided.

RESULTS

To determine whether K1 might play a role in the clinical manifestations of KS, we analyzed human KS for the presence of K1 RNA. RT-PCR was performed on total RNA isolated from a KS lesion from an HIV-1-infected individual. RT-PCR analysis showed a product of approximately 280 base pairs (bp), and the band was not generated by PCR after treatment of the template with ribonuclease (RNase) A (Fig. 1, A). The band remained after treatment of the templated RNA with deoxyribonuclease (DNase) I (not shown). RNA isolated from 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated BC-3 cells (reported to contain approximately 40 viral genomes per cell during latency) used as a positive control showed a more intense signal. Thus, K1 is expressed in human KS, and TPA enhances its RNA levels in cell lines. These results, combined with recent evidence that HHV8-infected humans develop cytotoxic T-cell lymphocyte responses targeting K1 (42), indicate that K1 is expressed at the protein level in the course of HHV8 infection.

Because viral gene expression is anticipated to be tightly regulated, particularly in chronic viral infection, and viral transcripts are produced, we attempted to stimulate and characterize K1 gene expression of latent HHV8-infected cells. Cellular RNA of TPA-treated KS-1 and BC-3 cells was subjected to northern blot analysis with the use of K1 DNA as the labeled probe. Transcripts of 1.3 and 3.6 kb were observed to be either induced or enhanced with TPA treatment (Fig. 1, B). The smaller band corresponds to the length of a K1 transcript, whereas the higher molecular weight transcript likely represents a complex transcript encompassing K1 (43,44). Ethidium bromide staining of 28S ribosomal RNA served to indicate the relative levels of RNA loading. Thus, K1 is expressed in these cells, consistent with lytic-phase gene expression *in vitro* and by extrapolation during lytic-phase HHV8 expression in human KS.

Of the HHV8 genes considered as candidates for mediating inflammation in KS, K1 is a promising candidate. The K1 protein sequence exhibits a highly conserved cytoplasmic ITAM that is involved in inflammation (45,46). We cloned K1 (GenBank accession No. AF170531) from the primary effusion lymphoma-derived cell line BC-3, which is chronically infected with

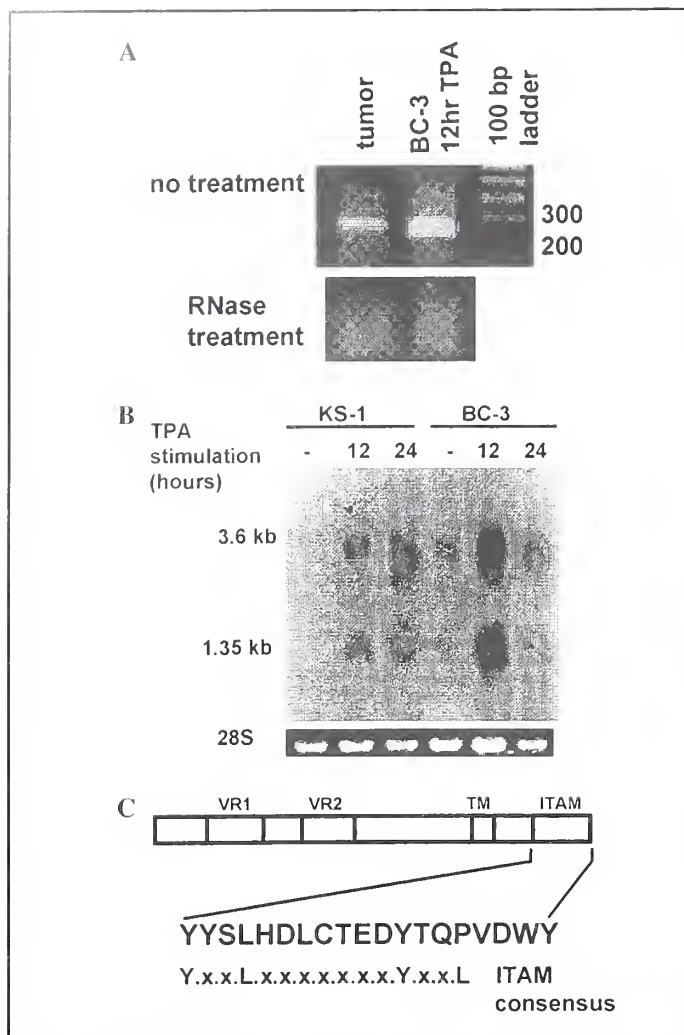


Fig. 1. K1 from BC-3 cells was cloned and contains an immunoreceptor tyrosine-based activation motif (ITAM) (YxxLxxxxxxYxxL). **A)** Kaposi's sarcoma (KS) tumor and cell lines express K1 RNA. Reverse transcription-polymerase chain reaction was performed on total RNA isolated from KS tumor. DNA was separated on agarose gel and stained with ethidium bromide. Treatment of RNA template with ribonuclease (RNase) prevented the synthesis of DNA product, whereas the treatment with deoxyribonuclease allowed synthesis of DNA product. **B)** Human herpesvirus 8-infected BC-3 and KS-1 cells express K1 when stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The smaller band corresponds to the transcript of K1, and the larger band is likely a multigene transcript that includes K1. **C)** K1 from BC-3 primary effusion lymphoma protein sequence predicts a transmembrane protein with extracellular highly variable regions (VR1 and VR2), a transmembrane (TM), and a short cytoplasmic tail with an ITAM (YxxLxxxxxxYxxL), which plays an essential role in host signaling pathways. The amino acid sequence is for clades A and C. bp = base pair; kb = kilobase.

HHV8 (*see below*). The predicted protein sequence from the K1 DNA from BC-3 cells reveals a transmembrane domain protein with an extracellular immunoglobulin light chain-like domain, two hypervariable regions (29), and a short cytoplasmic tail that contains an ITAM (YxxLxxxxxxYxxL) (Fig. 1, C). Amino acid sequence shared by clades A and C is shown, and the ITAM is identified. The rightmost leucine (L) is substituted by proline (P) in K1. K1's highly variable protein sequence can be classified into several clades A–D (BC-3 is C); however, thus far, a clade-specific clinical entity or pathology has not emerged (29).

K1 DNA from BC-3 cells was tagged at its cytoplasmic car-

boxy terminus with a myc epitope that is targeted by 9E10 antibody. Also, to speed up the evaluation of K1 function, we performed initial studies on Cos-1 cells because of their relative ease of transfection and their reproducibly high level of expression of plasmids. These studies serve as the basis for further studies with KS-derived spindle cells (38). Expression of the tagged protein was confirmed by detection of K1myc protein in transfected cells (Fig. 2, A). Transfectants were stained with anti-myc and fluorescein isothiocyanate-conjugated secondary antibody, which localized activity to cell membrane in K1myc transfectants but showed no staining in vector transfectant controls (not shown). Transfection with a plasmid-directing expression of a cytosolic protein β catenin-myc showed activity in a different distribution and predominantly in dividing cells (Fig. 2, B).

For the investigation of a possible role for the ITAM-containing HHV8 K1 protein in inducing inflammation, K1 was expressed in Cos-1 cells, and cell extracts were analyzed for NF- κ B-dependent promoter activity (Fig. 3, A). Cells expressing K1myc showed NF- κ B-driven luciferase activity of 19200 U and its vector control transfectant level of 4400 U (Fig. 3, A). Of interest, no cross-linking reagents were necessary for K1 to generate NF- κ B-driven expression. Cells expressing MEKK displayed enhanced luciferase activity, whereas mock transfectants displayed luciferase activity comparable to vector transfectants (not shown). Transfection of plasmid-directing expression of green fluorescent protein showed equivalent cell transfection rates in K1 versus vector transfectants, indicating that the levels of transfection were similar regardless of the plasmid used. Transfection of cells with K1 that lacked a myc-tag produced similar NF- κ B-dependent activation, indicating lack of interference from the myc epitope (not shown).

To determine if another NF- κ B-related promoter could be activated by K1, we coexpressed K1 with plasmid containing luciferase driven by a promoter containing AP-1 sites. K1 expression showed a greater than twofold increase in AP-1-dependent luciferase activity (Fig. 3, B). To begin analyzing the effects of K1 expression in KS cells, we transfected pCR3.1K1myc into KS Y-1 cells along with the luciferase re-

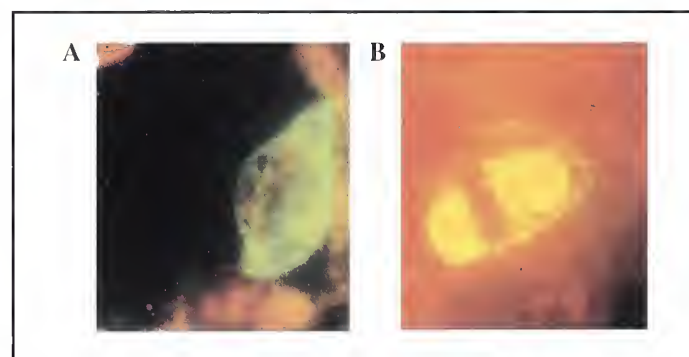


Fig. 2. K1 appears to be a membrane-associated protein. **A)** Cos-1 cells transfected with pSG5K1myc and stained with the anti-myc and fluorescein isothiocyanate anti-mouse antibodies show distribution of K1 with cell membrane. Cos-1 cells transfected with pSG5 lack any fluorescence activity (not shown). The staining pattern of K1 is consistent with the distribution of protein associated with cytoplasmic membrane. **B)** Staining of Cos-1 cells transfected with a plasmid expressing the cytosolic protein β catenin-myc displayed a different distribution in cells, which also displayed reduced cell spreading and was seen usually in dividing cells. Nuclei were visualized with Evans blue staining. Original magnification $\times 400$.

Table 1. Cytokines secreted by K1 transfectants*

Cytokine	Mean \pm standard deviation	
	Vector transfectants, pg/mL	K1 transfectants, pg/mL
GM-CSF	66 (± 8.5)	171 (± 11.3)
IL-6	1587 (± 18)	1870 (± 42)
IL-8	32 (± 28)	123 (± 59)
IL-12	12 (± 1)	132 (± 5)

*Cells were transfected with pCR3.1K1myc or pCR3.1, and cell supernatants were collected at 24 hours. The cells' conditioned media were analyzed for human cytokines by enzyme-linked immunosorbent assay (ELISA). Results are reported from Kaposi's sarcoma (KS) Y-1 transfectants with the exception of the analysis of interleukin (IL)-8, which was done with Cos-1 cells with the use of ELISA for human IL-8. No statistically significant differences were noted in the levels of RANTES, monocyte inflammatory protein 1 (MIP-I), and MIP-II. The means of cytokine levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-8, and IL-12 of K1 transfectant supernatants were statistically significant (each $P < .05$).

Cos-1 cells also showed enhanced IL-8 secretion. These cytokines bear NF- κ B-response elements in their promoters (47). This cytokine production was not a generalized effect because K1 expression did not induce elevation of other inflammatory cytokines, such as RANTES, MIP-I, or MIP-II. The cytokines induced by K1 are inducible by cell stimuli that operate through NF- κ B (47), and the cytokines are also implicated in KS lesion formation (15-18,20,23).

We have hypothesized that the inflammation in KS lesions is mediated by a few lytically active cells that possess pervasive inflammatory effects on other cells. To evaluate the possibility of these paracrine effects, we examined the cells' conditioned media for their ability to promote NF- κ B-dependent activation. Cos-1 cells incubated with conditioned media of K1 transfectants exhibited enhanced NF- κ B-promoter activity, indicating that K1 mediates a paracrine effect (not shown). Taken together, the secretion of numerous cytokines and a second wave of NF- κ B activation would support the development of diffuse tissue inflammation present in KS lesions.

Activation pathways can be blocked by specific therapeutic anti-inflammatory or immunoregulatory agents, such as aspirin and cyclosporin (48,49). To determine whether aspirin or cyclosporin would block K1-triggered activation, we treated pCR3.1K1myc and pCR3.1 transfectants with either aspirin or cyclosporin for 24 hours after transfection. K1 transfectants demonstrated enhanced NF- κ B-dependent activity over buffer-treated vector transfectants (Fig. 4). Treatment of cells with aspirin blocked the K1-dependent promoter activity down to the activity of vector-alone transfectants, indicating selective blockage of inducible luciferase activity. Treatment of cells with cyclosporin also abrogated the K1-induced luciferase activity (Fig. 4). However, in contrast to aspirin treatment, the vector transfectants treated with cyclosporin also showed significantly lower luciferase activity, indicating that baseline activity was also affected. Thus, drugs that block inflammation and cell activation demonstrate the ability to block inflammation-related K1 signaling and further substantiate that K1 signals via host NF- κ B-dependent pathways.

Because expression of the cell membrane-associated K1 activates NF- κ B in the absence of an added ligand and viral receptors generally mimic activated host receptors, we surmised that K1 from BC-3 might be constitutively activated. ITAM-

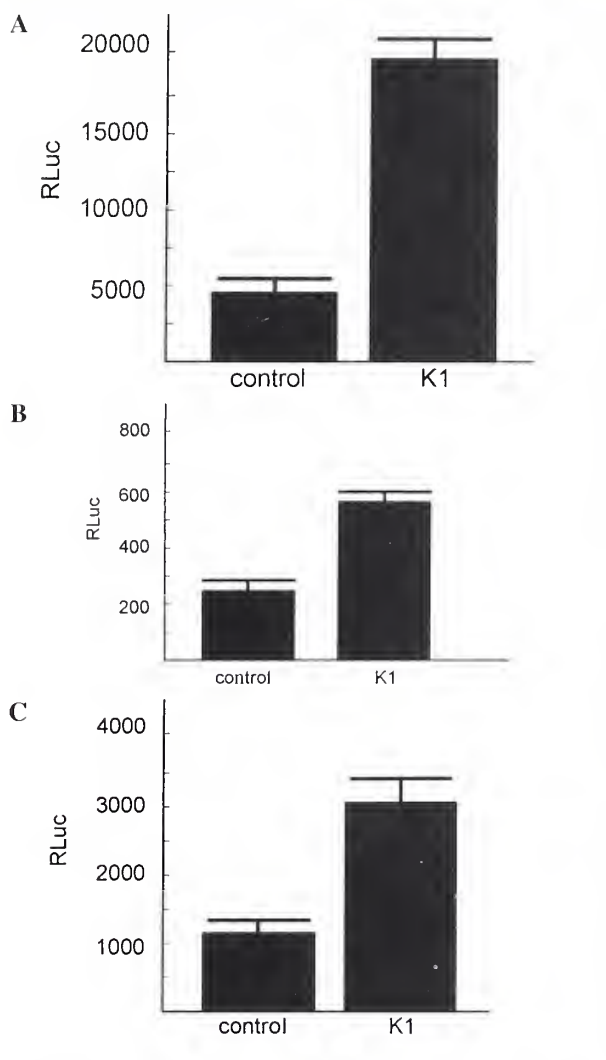


Fig. 3. Expression of K1 stimulates nuclear factor-kappa B (NF- κ B)-dependent transcription. Cos-1 cells were transfected with the use of pCR3.1K1myc or pCR3.1 with pNF- κ BLuc and Fugene (1 μ g DNA/ 10^7 cells); 24 hours later, cell extracts were analyzed for luciferase activity. **A)** The results are the mean and standard deviation of each cell from three wells of six-well plates. Expression of green fluorescence protein indicated no differences in transfection efficiencies. Transfection with control positive pFCMEKK produced enhanced luciferase activity. **B)** Expression of K1 induces AP-1-dependent activity in Cos-1 cells. **C)** Transfection of KS Y-1 cells with pCR3.1K1myc and pNF- κ BLuc was analyzed for luciferase activity. **Bars** indicate the means and standard deviation from different transfection pools. RLUC = relative light units concentration.

porter construct. NF- κ B-dependent luciferase activity in K1 transfectants was three times the activity observed in vector (control) transfectants (Fig. 3, C). In all cases, K1-stimulated activity was consistently higher than vector transfectant controls. Thus, K1 stimulates transcriptional activity at two promoter/enhancer sites, AP-1 and NF- κ B, and suggests that KS cells are competent to handle ITAM-related signaling.

NF- κ B plays a central role in the induction of numerous immunoregulatory responses, including expression of IL-1, IL-2, IL-3, IL-6, IL-8, tumor necrosis factor- α , and interferon gamma. KS Y-1 cells transfected with K1 compared with vector transfectants showed 11-fold higher levels of IL-12 and significantly higher levels of IL-6 and granulocyte-macrophage colony-stimulating factor ($P < .05$) (Table 1). K1-transfected

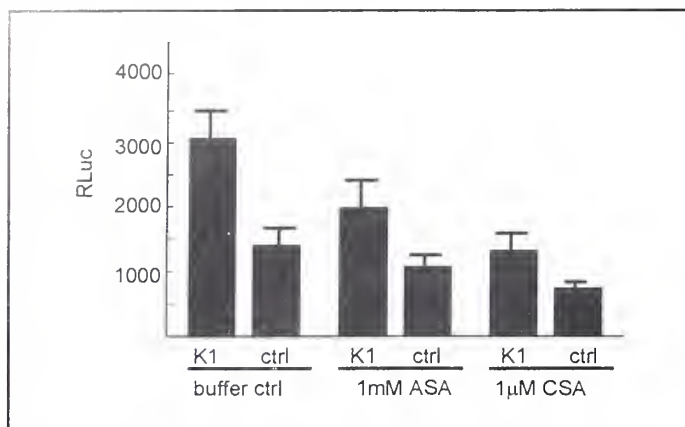


Fig. 4. Signaling of K1-triggered nuclear factor-kappa B (NF- κ B) pathways can be blocked by reagents that block cell activation and inflammation. Cos-1 cells were transfected with pCR3.1K1myc or pCR3.1 with pNF- κ BLuc and incubated with growth media containing aspirin (ASA) or cyclosporin (CSA). After 24 hours, cell extracts were made and the luciferase assay was performed. RLUC = relative light units concentration; ctrl = vector alone transfect units.

receptor binding and/or aggregation is typically required for phosphorylation of ITAMs that constitutes an activated state. Therefore, we examined K1 for phosphorylation by combined immunoprecipitation/immunoblot analysis by transfecting Cos-1 cells with pSG5K1myc and detection with anti-myc antibody. Our studies show that K1 appeared as a protein of approximately 47 kilodaltons with apparent lower molecular weight diffuse bands (Fig. 5). K1 can be glycosylated, and the lower molecular weight bands likely represent incompletely glycosylated forms of K1 (50). No apparent multimers of K1 were observed in contrast to other studies (30,50). The bases for this difference may rest on the different clades of K1 or on the differences in methods of detection. Similar immunoprecipitation/immunoblot of β catenin-myc transfectants showed an expected band of β

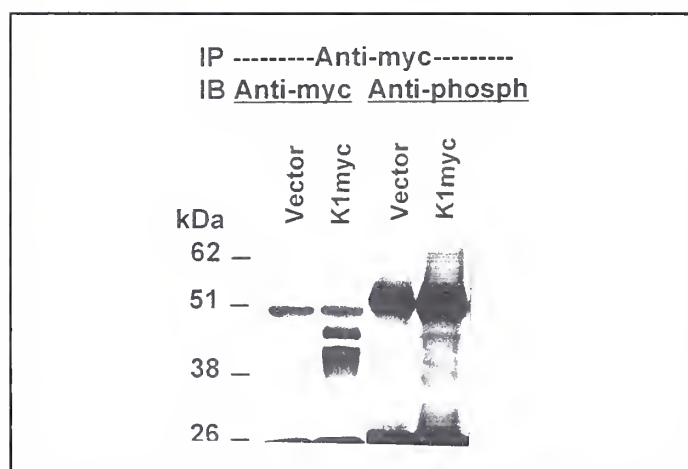


Fig. 5. K1 is expressed as a constitutively phosphorylated protein. Cos-1 cells were electroporated with pSG5K1myc or pSG5, and extracts were subjected to combined immunoprecipitation and reducing SDS-PAGE. Immunoblotting of filters were performed with anti-myc antibody and a duplicate filter blotted with anti-phosphotyrosine (anti-phosph) antibody. A 47-kilodalton (kDa) band is recognized by anti-myc antibody as well as less intense and lower molecular weight forms. The antiphosphotyrosine (4G10) blotting shows phosphoprotein of 47 kDa. An anticipated band of approximately 80 kDa appeared on immunoprecipitation/immunoblot of Cos-1 cells transfected with pSVK3- β catenin-myc (not shown).

catenin-myc, and vector-transfected Cos-1 cells lacked either of these bands (not shown). To determine whether the immunoprecipitated K1myc was also phosphorylated, we blotted a duplicate filter with anti-phosphotyrosine antibody and noted the appearance of a phosphoprotein approximating the size of the largest K1 band (Fig. 5). There was no phosphorylation signal in the region immediately below the K1 band, even after extended exposure of the film. Of interest, only the high-molecular-weight K1 band was phosphorylated (at the present level of detection), suggesting that only the completely processed K1 (glycosylated) is preferentially phosphorylated. This finding indicates that K1 that is myc-tagged can serve as a substrate for phosphorylation and is active in NF- κ B-dependent transcription. Thus, even in the absence of a ligand, K1 is expressed as a phosphorylated protein, consistent with its being a constitutively activated protein capable of signaling through ITAM-dependent pathways.

Although HHV8 infection alone, without intercurrent illnesses, is sometimes associated with milder forms of KS, the presence of a second virus, i.e., HIV-1, usually dictates an aggressive clinical course of KS that parallels the activity of HIV-1. HIV-1 appears to stimulate KS cell proliferation indirectly by inducing inflammatory cytokine production, and this production depends, in part, on NF- κ B-dependent promoters (51). To assess for a possible contribution of HIV-1 Tat to the ability of K1 to stimulate NF- κ B-dependent transcription, we coexpressed K1 and Tat (52). Transfection of pCR3.1K1 or pCMVtat stimulated NF- κ B-dependent luciferase activity of 490 and 380 arbitrary units, respectively, over vector-alone transfectants (Fig. 6). However, transfection with the combination of plasmids showed enhanced promoter activity in an additive fashion. Thus, in this model, two genes from disparate origins converge on NF- κ B-dependent transcriptional activity in a cooperative fashion. In this regard, HIV-1 and HHV8 share signaling targets to bring about a cellular inflammatory response at a level not observed with each viral product acting alone.

DISCUSSION

The variable clinical course of KS and the histologic features of KS lesions suggest that the inflammatory process is central to the promotion of KS lesions. HHV8 may be the stimulus for

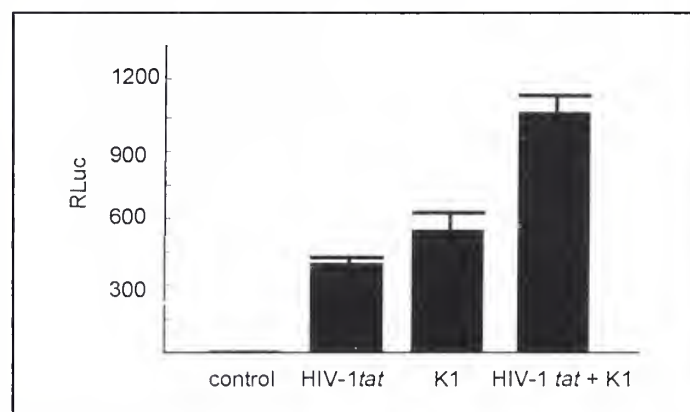


Fig. 6. K1 and human immunodeficiency virus type 1 (HIV-1) Tat cooperate in activating nuclear factor-kappa B (NF- κ B)-dependent transcription. Cos-1 cells were cotransfected with plasmids encoding for both proteins. Each plasmid alone shows enhanced NF- κ B-dependent promoter activity, and in combination the promoter activity was increased over single plasmid transfectants in an additive fashion. RLUC = relative light units concentration.

inflammation and, to that end, we have presented data implicating HHV8 K1 in activating pathways that operate in cell activation and inflammation. K1, like other ITAM-containing proteins, may provide cells with a critical signal that ultimately determines cell activation, at least, in part, by inducing NF- κ B- and AP-1-dependent promoter activity.

The ITAM from K1 can transmit signals when tested as a chimeric protein joined to the extracellular domain of a known receptor (CD8) (31). Even though signaling through ITAM can transmit inhibitory signals that are dependent on context, K1 ITAM fused to CD8 positively stimulated signaling (31,45,53,54). More recently, full-length K1 from body cavity-based lymphoma-1 cells (clade A3) was shown to induce NFAT-dependent promoter activity (30,50). NFAT is another ITAM-dependent factor described in lymphocytes that regulates promoter-driven expression of proteins associated with inflammation and proliferation (55). Thus, K1 leads to activation of NFAT- and NF- κ B-dependent promoter activities that, in turn, orchestrate the transcription of an array of proteins involved in cell activation and inflammation.

K1 is likely to activate pathways used by host cell ITAM-containing proteins. However, unlike the K1 protein product, which is constitutively active, host cell ITAM-containing proteins generally reside in the resting state, and, on ligand binding or receptor aggregation, they undergo phosphorylation and become competent for signaling. ITAM-containing receptors include subunits of the B-cell receptor, T-cell receptor, and Fc receptor, which contain one or more copies of ITAMs within their cytoplasmic tails (56). Specifically, these ITAM-bearing proteins include human proteins TCR- ζ , CD3 δ , and Fc ϵ R1 and viral proteins, such as HHV8 LMP2A-like protein, Hantavirus glycoprotein G1, bovine leukemia virus gp30, Epstein-Barr virus LMP2A, and rhesus monkey rhadinovirus R1 (32,45,53,57–61).

NF- κ B-dependent signaling is modulated by viral proteins as well as by ligands and drugs that affect inflammation and cell activation. HIV-1 Tat is shown to contribute to NF- κ B-dependent promoter activity that is additive to that of K1. Because HIV-1 infection substantially accelerates the aggressive course of KS, additive effects at NF- κ B-dependent promoter activity may be one key mechanism in which HIV-1 and HHV8 converge to stimulate cell activation and inflammation and account for an aggressive course of KS in HIV-1 infection (62).

Although lymphocytes were first discovered and extensively studied for expression of ITAM receptors, other cells are known to harbor functional ITAM-bearing receptors as well. Indeed, monocytes and macrophages (MO/MC) contain ITAM-containing receptors, such as the immunoglobulin Fc receptors. In MO/MC, ligand binding activates Fc receptors to induce cell activation by expression of the IL-2 receptor and secretion of inflammatory cytokines (tumor necrosis factor- α and IL-12), which together can synergize in mounting a greatly amplified inflammatory response (63,64). The copious cytokines released in K1-expressing cells would predict that K1 expression in MO/MC would mount a wave of cytokine secretion and would contribute toward an overall activation of MO/MC. In KS tissue, despite the fact that most cells contain HHV8 in the latent phase, some lytic viral replication does occur in cells that share markers of activated MO/MC and ECs (CD68) (65). These cells are expected to express K1 as part of their lytic-phase expression program that would be implicated in KS inflammation (Fig. 7).

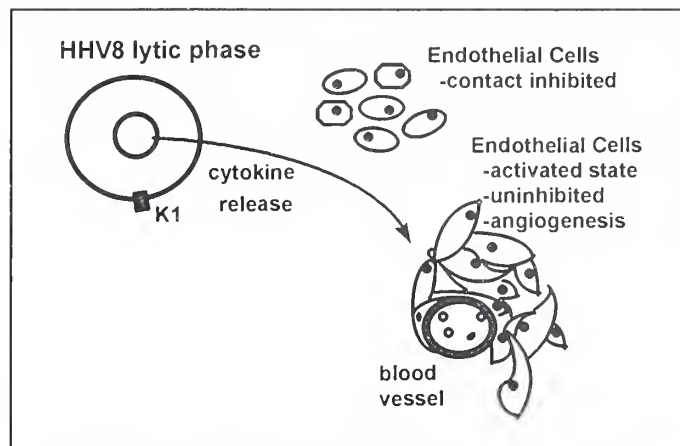


Fig. 7. Expression of human herpesvirus 8 (HHV8) K1 stimulates signaling pathways involved in cell activation and cytokine production. Cell signaling through immunoreceptor tyrosine-based activation motif-containing proteins induces activation of the cells and also induces secretion of inflammatory cytokines. K1 expressed in lytically active cells would contribute to inflammation that includes secretion of inflammatory cytokines. Inflammatory cytokines induce endothelial cells to acquire features of Kaposi's sarcoma spindle cells that include angiogenic factor production, expression of adhesion proteins, and a secondary wave of inflammatory cytokine production.

MO/MC, in particular the subendothelial MO/MC, have been noted to express substantial levels of activation markers, and their location in the subendothelium suggests that they play pivotal roles in transluminal trafficking.

The small percentage of cells in KS lesions that undergo lytic-phase replication are anticipated to express K1 and to dictate the inflammatory status of KS tissue. Therapy targeting herpesvirus in humans leads to regression of KS and lowers the frequency of KS development (66). Antiviral therapy in experimental models has reduced the levels of HHV8 in human SCID mouse models infected with HHV8 (14,33). In our cell model, we show that K1 stimulates NF- κ B and that agents known to block inflammation or cell activation also block K1-mediated NF- κ B-dependent promoter activity. Thus, the link between inflammation and HHV8 gene regulation and replication appears as a central event to the development of KS. In summary, K1, which functions by mimicking the activity of host ITAM proteins, is likely to be a major trigger for cell activation and inflammation in KS.

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NOTES

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Hematopoietic Stem Cells in HIV Disease

David T. Scadden, Hongmei Shen, Tao Cheng

The hematopoietic stem cell has long been hypothesized to be a target of human immunodeficiency virus type-1 (HIV) infection that limits the potential for compensatory immune cell production. Data have recently emerged documenting stem cell dysfunction in HIV disease and indicating that immune recovery from potent antiretroviral therapy is partly driven by new T-cell generation. Effects of HIV on stem cell physiology, however, appear to be indirect, as stem cells are highly resistant to HIV infection. Despite the presence of surface receptors for HIV, the hematopoietic stem cell is not infectible with HIV. However, stem transduction can be achieved with HIV constructs in which the envelope glycoproteins have been replaced by vesicular stomatitis virus G protein. Therefore, hematopoietic stem cells are likely participants in HIV-related cytopenias, but they are spared direct infection and can serve as a resource for cellular therapies for AIDS. [J Natl Cancer Inst Monogr 2000;28:24-9]

Human immunodeficiency virus (HIV) induces a multitude of alterations in the innate and adaptive immune system that are broad if not equally distributed among virtually every arm of host defense. Because T cells are but one of the cell types affected, the potential for HIV being a disease of stem cells as well as mature effector cells has long been hypothesized. If stem cell dysfunction or destruction plays an important role in HIV disease, the implications are multiple and include limiting immune reconstitution and affecting the potential for stem cell-based gene therapy strategies.

Possible mechanisms include direct virus infection or indirect effects by altering the cellular or cytokine milieu of the bone marrow microenvironment. The issue of direct infection will be addressed in a later section of this paper in which the marked resistance of stem cells to HIV-1 infection is discussed. The lack of infectibility of stem cells, however, is not synonymous with the lack of adverse effects of HIV because of direct interactions with the virus. The virus envelope glycoprotein, gp120, is capable of interacting with the CXCR-4 and of inducing intracellular signaling events as manifest by calcium flux, kinase activation, and even functional changes, such as chemotaxis of some cell types (1,2). Whether it may directly induce altered function of stem cells that express CXCR-4 is not clear, but data suggest that apoptosis may be induced (3).

Alteration in the cellular and cytokine milieu of the bone marrow has been reported by a number of investigators who have demonstrated infection of bone marrow stromal elements and perturbation of either cytokine production or ability to sustain hematopoiesis (4-6).

***In Vivo* Evaluation of Stem Cells in HIV Disease**

Stem cell functional defects *in vivo* have been demonstrated both in human studies or in animal models. In animal models in

which human hematopoietic tissue is engrafted in immunodeficient mice, reduced CD34⁺ cells and/or decreased colony-forming capacity have been shown to occur after HIV infection (7,8). Efforts to define the stem cell pool in HIV-infected patients have been less uniform in their conclusions, but an important study (9) quantitating circulating CD34⁺ cells after granulocyte colony-stimulating factor (G-CSF) mobilization has recently been concluded. These studies have shown that a decline of CD34⁺ numbers is seen with more advanced HIV disease. Patients with lower CD4 cells have lower concentrations of CD34⁺ cells after G-CSF mobilization. The stem cell numbers that can be harvested from patients with CD4 counts below 200 cells/mm³ may still be adequate for purposes of transplantation, but their concentration per milliliter of blood is demonstrably lower than those patients with more preserved immune function (9).

CELL KINETICS IN IMMUNE DECLINE AND REGENERATION

A causal relationship between lower CD4⁺ cells and CD34⁺ cells has been suggested by two recent lines of evidence. The first is the sequential analysis of stem cell and lymphocyte numbers in the previously mentioned mouse models. These studies have indicated that, after acute HIV infection, there is a decline in primitive cell numbers and function that precedes the decline in thymocytes (8). The second is related to the changes seen as viral replication decreases after antiretroviral therapy. Reduced HIV replication is associated with an improvement in CD34⁺ cells, myeloid colony-forming capacity, and CD4⁺ T cells (10). The increase in CD4⁺ cells is not related to improved T-cell survival but rather appears to be due to increased rates of production (11). The initial increase in CD4⁺ cells may be due to expansion of existing mature memory cells. However, with the use of immunophenotypic markers for naive cells and a recently developed polymerase chain reaction (PCR) method to quantitate cells recently emigrating from the thymus (12), it is clear that *de novo* generation of T cells is occurring (12-14). Although it has not been rigorously shown that the T-cell regeneration reflects stem cell changes, it is apparent that hematopoiesis rather than improved cell survival times is the driving force of immune reconstitution after antiretroviral treatment. The improvement in T-cell generation may then either reflect improved primitive cell function or number or the improved health of the tissue microenvironments in which the differentiation of primitive cells occurs.

Affiliation of authors: D. T. Scadden, H. Shen, T. Cheng, AIDS Research Center and Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston.

Correspondence to: David T. Scadden, M.D., AIDS Research Center and Cancer Center, Massachusetts General Hospital, 149 13th St., Rm. 5212D, Boston, MA 02129 (e-mail: scadden.david@mgh.harvard.edu).

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Disruption of thymic architecture has been well described with HIV infection (15–17), but recent data would suggest that the defects in thymic function are neither complete nor irreversible. A radiographically detectable thymus is present in many HIV-infected patients (18); and even in settings of severe dysfunction, T-cell production is occurring. HIV-infected individuals who have had thymectomies for myasthenia gravis have ongoing T-cell generation (19). Also, in the setting of antiretroviral therapy, thymic dysfunction appears to be at least partially reversible. *In vivo* models have shown improvement in T-cell generation from precursor populations both endogenous and exogenous to the thymus with accompanying control of viremia (13,20). The potential of thymic function is greater than previously thought, and abnormalities of function are likely to be at least partially reversible. Thus, the thymus is considered to be less likely to represent a significant limiting factor in determining the extent to which T-cell numbers will improve after antiretroviral therapy. Rather, the primitive cell pool or other differentiation regulators may provide the difference between those patients who improve to normal or near normal levels and those who do not.

STEM CELL SUSCEPTIBILITY TO HIV-1 INFECTION

The emphasis on the stem cell in the context of HIV disease is then shifting in several important ways. The first is recognition that stem cells may indeed play a role in restricting immune restoration. The second is that their longevity presents stem cells as a potentially long-lived reservoir for HIV if infected. And the third is the greater potential for stem cells as a therapeutic tool in the context of gene therapy. If the thymus is not limiting, genetically protected stem cells may indeed be capable of providing the substrate for T-cell generation. Crucial to each of these issues is the infectibility of the stem cell by either HIV-1 or lentivirus vectors derived from HIV-1.

HIV RECEPTOR/CO-RECEPTOR EXPRESSION IN HEMATOPOIETIC CELL SUBSETS

We recently completed studies in which cells representing particular stages of blood cell development were isolated by flow cytometric or functionally based systems (14,21–23). The stem cell population was isolated with the use of the previously described method of selectively killing more mature cells thereby enriching for a cytokine nonresponsive subset with stem-like characteristics. Cells were assessed for expression of CD4, the chemokine receptors (CCR-5 and CXCR-4), messenger RNA, and protein with the use of the techniques adapted for the small numbers of primary cells available from standard donations. Low levels of message for CD4 were detectable by reverse transcription (RT)-PCR in peripheral blood mononuclear cells (PBMCs), myelomonocytic cells (CD11b⁺), mature T cells (CD3⁺CD4⁺), heterogeneous hematopoietic progenitor cells (CD34⁺), lineage-committed hematopoietic progenitor cells (CD34⁺CD38⁺), primitive hematopoietic progenitor cells (CD34⁺CD38⁻), and stem cells (G₀), but not in NIH3T3 control cells. Similarly, the message for CXCR-4 and CCR-5 was detectable in each hematopoietic cell type tested, although CCR-5 levels in CD34⁺CD38⁻ cells appeared to be lower

as confirmed on multiple independent samples (*n* = 4). To more precisely define the presence of the receptor transcripts in stem cells, individual cells were isolated by micromanipulation, and single-cell RT-PCR profiles were generated as described previously (24,25). The cells consistently demonstrated detectable CD4, CXCR-4, and CCR-5 messages compared with control cells.

The presence of protein produced from the receptor transcripts was assessed by specific antibody staining and, independently for chemokine receptors, by calcium flux. Anti-CD4 staining analyzed by flow cytometry indicated CD4 expression in subpopulations of CD34⁺ cells similar to the findings reported by others (3,26–28). CCR-5- and CXCR-4-specific antibodies stained fractions of relevant CD34⁺ cells with only minimal staining of CD34⁺CD38⁻ cells with the use of the anti-CCR-5 antibody, consistent with the low transcript levels observed.

Chemokine signaling, as measured by the generation of a calcium flux in cells bearing cognate receptors, was used as a functional assessment of chemokine receptors. Responsiveness of various subsets of CD34⁺ cells to SDF-1 (ligand for the CXCR-4 receptor) and regulated-on-activation normal T cells expressed and secreted (RANTES), MIP-1a, and MIP-1b (ligands for the CCR-5 receptor) was measured on Indo-1-loaded cells with the use of the fluorescence-activated cell sorter analysis. NIH3T3 cells were used as a cell control, IP-10 (the ligand for CXCR-3) was used as a chemokine control, and measurements were made over time by using the cells before and after exposure to chemokine to establish a target-cell baseline control. Response to SDF-1 was substantial in all populations of CD34⁺ cells, although increased response was noted in the CD34⁺CD38⁻ cells, despite no difference in the frequency of CXCR-4 surface protein in that subfraction compared with CD34⁺CD38⁺ cells. Similarly, the relationship between detectable surface protein for CCR-5 and response to ligand was not direct. Despite low levels of message and surface CCR-5 in the CD34⁺CD38⁻ subset, calcium flux was approximately equivalent to other cell fractions when cognate ligands were applied. NIH3T3 cells did not demonstrate calcium flux, and IP-10 did not induce calcium flux except in a huCXCR-3-expressing cell line.

The rarity of stem cells (G₀) precluded the routine use of flow cytometry, and, thus, we developed an immunomagnetic bead rosette assay (Fig. 1). This assay uses the binding of specific monoclonal antibodies to target epitopes on cells similar to immunofluorescence assays. However, instead of fluorescein conjugation, immunomagnetic bead conjugation was used as a means of enhancing the ability to detect antibody binding with the use of microscopy; the size of Dynal beads permitted ready enumeration of rosetted cells and had a low frequency of non-specific binding (0.7%–6%) when second-step alone or when irrelevant antibody-conjugated beads were used. Estimated frequency of CD4-, CXCR-4-, and CCR-5-expressing cells compared with bead alone or with irrelevant antibody-conjugated bead controls (specific–nonspecific binding) was 12.2%, 23.2%, and 23.6%, respectively. Large-scale stem cell preparation generated by pooling multiple independent marrow preparations permitted flow cytometry analysis of CD34⁺ and CD4⁺ cells and demonstrated that a high fraction of these cells co-expressed CXCR-4 and CCR-5, compared with isotype control (Fig. 2).

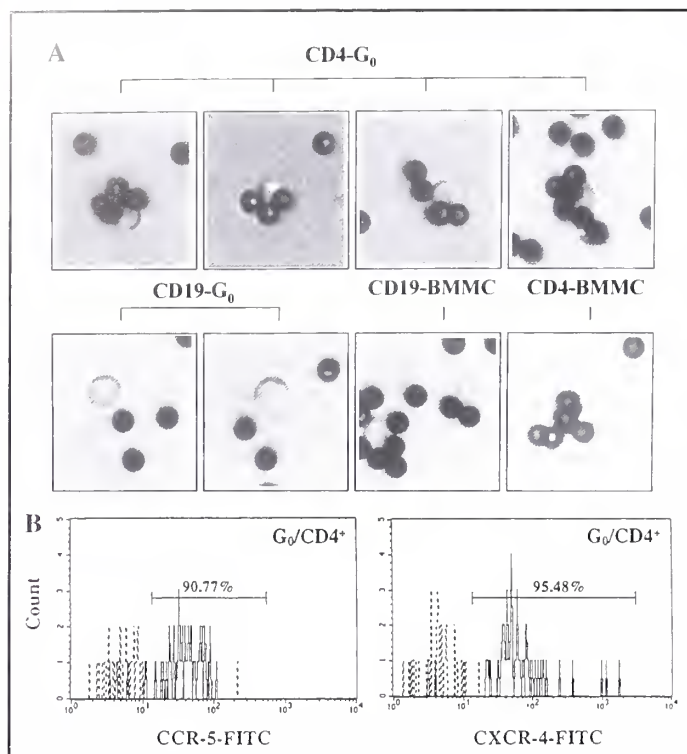


Fig. 1. Analysis of protein expression on stem cells was performed by using an immunomagnetic bead rosetting assay. Immunomagnetic beads (dark circles) were assessed for their binding to target cells by two independent readers with the use of phase-contrast microscopy (original magnification $\times 40$). The **upper panel** demonstrates representative CD4-specific beads binding to G₀ stem cells. The **middle panels** demonstrate lack of CD19-bead binding to G₀ stem cells (negative control) or beads binding to peripheral blood lymphocytes (positive controls). **Lower panel:** G₀ cells selected for CD4 were pooled, and flow cytometrically analyzed for staining with either CXCR-4 or CCR-5 or isotype controls.

Single-cell digital fluorescence imaging was used to document stem cell (G₀) calcium flux in response to chemokines. MIP-1a, MIP-1b, and SDF-1 generated evidence of calcium flux (Fig. 2, B) and thereby confirmed the functional status of surface CCR-5 and CXCR-4 on stem cells. In contrast, IP-10 did not

induce flux, whereas inducing calcium flux in huCXCR-3 transduced control cells.

HIV-1 CO-RECEPTORS FUNCTION ON CD34⁺ CELLS BUT NOT G₀ STEM CELLS

Definition of the functional characteristics of the co-receptor molecules was further pursued through exposure of cells to stocks of infectious HIV-1. Given the presence of identifiable receptors for M-tropic strains (with the use of CCR-5) and T-tropic strains (with the use of CXCR-4), appropriate virus envelopes were used (HIV-1_{Ba-L} and HIV-1_{HXB-2}, respectively). Following exposure to concentrated stocks of virus, infection was evaluated for 1) the presence of HIV DNA, indicating virus entry and RT, and 2) the production of HIV-1 p24 antigen after addition of highly infectible cell lines, indicating completion of a replicative virus life cycle and passage of the virus. Virus DNA was detectable at the level of a single cell diluted to 10^{-4} in titration experiments of ACH-2 cells that contained a single proviral copy per cell (data not shown). HIV-1 DNA was evident in all subsets of cells exposed to infectious, but not heat-inactivated virus, with the notable exception of the G₀ stem cells (Fig. 3, A). G₀ cells independently isolated from independent normal donors (three are shown) were consistently negative for viral DNA. CD34⁺-cell fractions other than stem cells had detectable viral DNA that was inhibitable at a level of approximately 50% by preincubation with cognate ligands for CCR-5, MIP-1a, MIP-1b, and RANTES (data not shown). The non-stem cell fractions also demonstrated productive completion of the virus life cycle by passage of HIV-1 to the readily infectible indicator cell lines PM-1 (Fig. 3, B) or H9 (data not shown) when HIV-1_{Ba-L} or HIV-1_{HXB-2}, respectively, were used. Prompt production and passage of the virus to PM-1 was noted from the relatively mature CD34⁺CD38⁺ population of cells. Although CD34⁺CD38⁻ cells had clearly identifiable virus DNA present, passage of infectious virions was very much delayed with p24 antigen detectable on co-cultivation with PM-1 only after prolonged periods (approximately 21 days). In no instance was p24 detectable on co-cultivation of indicator (PM-1 or H9) cells with virus-exposed G₀ cells, including experiments extended out to 35 days.

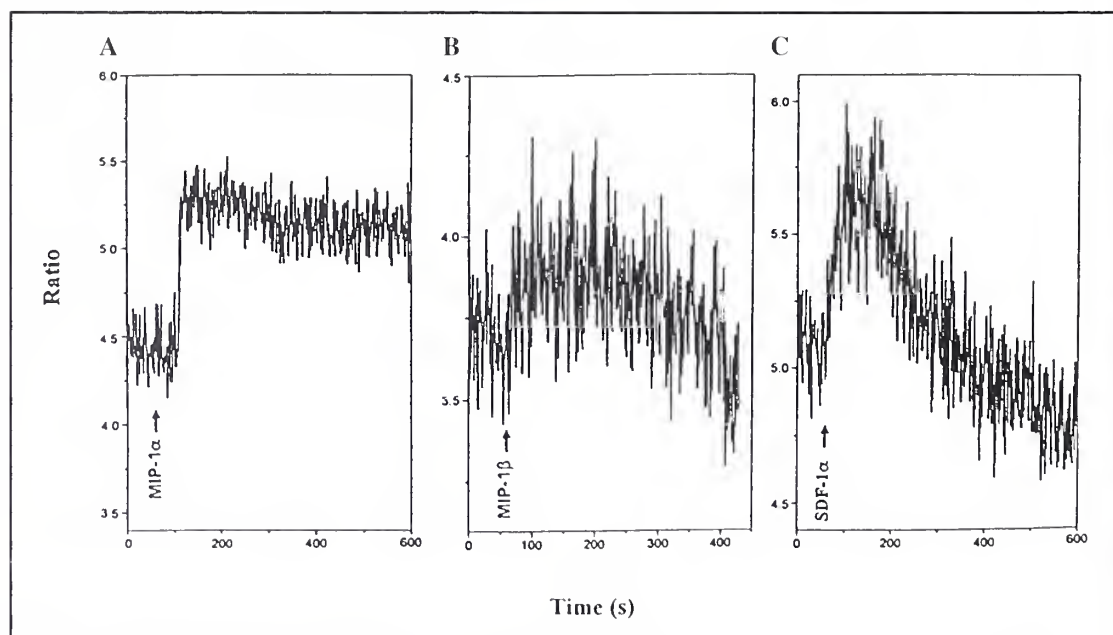


Fig. 2. Calcium flux in G₀ stem cells after stimulation with MIP-1a (A), MIP-1b (B), or SDF-1 (C) as assessed by laser-based digital fluorescence microscopy with quantitative graphic depiction of the fluorescence increase over time in fields of approximately 10 cells each is shown. Figures 2, B and C reprinted with permission by the American Society for Microbiology.

STEM-CELL RESISTANCE MEDIATED AT THE LEVEL OF VIRUS FUSION AND ENTRY

RT in quiescent cells may be incomplete and, in lymphocytes, may result in partial complementary DNA intermediates (29). To evaluate this possibility in stem cells, PCR primer pairs corresponding to 5' of the primer binding site and U3-U5 portions of the HIV-1 genome reverse transcribed even in quiescent lymphocyte populations were used (labeled TC-1 in Fig. 5). To assess if the level of block to infection was at the receptor level or following receptor interaction, an HIV-1 green fluorescent protein (GFP)-encoding construct pseudotyped with either T-tropic (HXB2), M-tropic (YU-2), or dual tropic (89.6) HIV-1 envelopes were compared with the same virus construct pseudotyped with the vesicular stomatitis virus G (VSV G) protein. VSV G permits virus fusion with the cell membrane via mechanisms that bypass those mediated by CD4 and CCR-5 (30). Only the VSV G-pseudotyped virus was capable of infecting the stem cell population (Fig. 5, A). No HIV DNA was detectable in stem cells when HIV-1 envelopes or heat-inactivated VSV G-envelope pseudotypes were used as assayed by either PCR or for GFP expression by fluorescence microscopy. HIV-1 envelope pseudotypes were capable of infecting Jurkat or primary mononuclear control cells. These data demonstrate that stem cells have a block to HIV-1 infection and that the level of the block is in the steps of viral-cell membrane fusion and entry. Steps downstream of these events are intact as evident from the VSV G-pseudotype infection.

An independent stem cell purification process with the use of Rhodamine 123 and Hoechst 33342 staining as defined by others (22) was used to exclude the possibility that the selection method induced alterations in the ability of the stem cells to be infected. The exclusion of Rhodamine 123 and low-intensity staining with Hoechst 33342 in CD34⁺ cells has been shown to associate with a population capable of functioning as stem cells in *in vitro* and *in vivo* experiments (31). After exposure to the virus, the cells corresponding to a more mature population (bright/bright) acquired detectable HIV DNA, but stem cells (dim/dim) had neither late nor early RT products detectable (Fig. 5, B) identical to what was seen with G₀ cells. Furthermore, these cells were readily infectible with the virus when the envelope was VSV G. These data confirm the resistance of stem cells to HIV-1 infection and demonstrate that the block can be overcome if an alternative CXCR-4- and CCR-5-independent mechanism of envelope-cell membrane fusion is used.

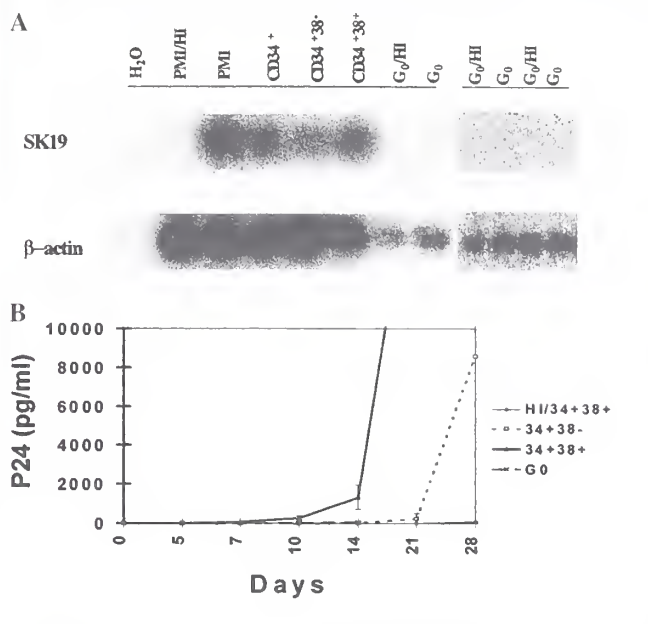


Fig. 3. HIV-1 entry and production is subset specific in hematopoietic cells. **A)** M-tropic human immunodeficiency virus (HIV)-1_{Ba-L} infection *in vitro* detected by DNA polymerase chain reaction (PCR) in indicated cell types. Pools of approximately 200 cells were assessed for HIV-1 DNA. Three independent preparations from independent donors are shown for the G₀ subset of cells. Consistent results were obtained from independent replicates-quadruplicates of all lanes shown. **B)** HIV-1 virus production in CD34⁺ subsets and G₀ stem cells that were exposed to HIV-1_{Ba-L} for 24 hours, were extensively washed, and subsequently co-cultured with PM1 cells as an indicator cell line, HIV-1 p24 assays were performed by enzyme-linked immunosorbent assay on culture supernatants at the indicated times. Similar results were obtained when identical experiments were performed by using HIV-1_{IIIb} with H9 cells as the indicator cell line. Figure 3. A reprinted with permission by the American Society for Microbiology.

IN VIVO CORROBORATION THAT STEM CELLS ARE RESISTANT TO HIV-1 INFECTION

To determine if stem cells were infected *in vivo*, bone marrow samples were obtained from HIV-1-infected patients with high levels of circulating virus and with low blood cell counts. With the use of multiple independent patient samples, HIV DNA was identified in PBMCs and bone marrow mononuclear cells (BMMC), but there was consistently no detectable HIV DNA in G₀ cells (n = 7) (Fig. 4).

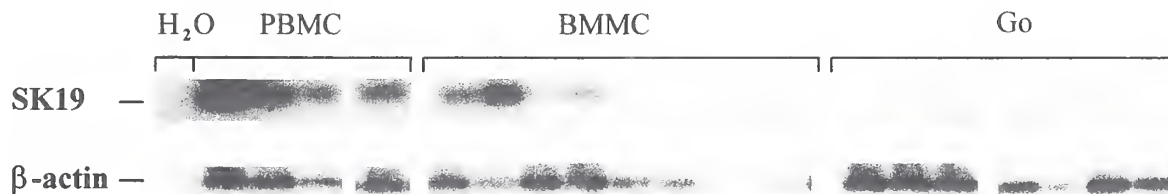


Fig. 4. Detection of human immunodeficiency virus (HIV)-1 genome in hematopoietic cells from AIDS patients as assessed by HIV-1 DNA polymerase chain reaction (PCR) analysis on subsets of cells derived from individuals with advanced HIV disease and cytopenia. **Each lane** represents cells from an individual patient. All G₀ lanes correspond to samples in the BMMC lanes with the

exception of one patient in which an inadequate number of BMMC cells were obtained to proceed with G₀ isolation. Peripheral blood mononuclear cell samples were obtained from independent patients with similar clinical profiles. Reprinted with permission by the American Society for Microbiology.

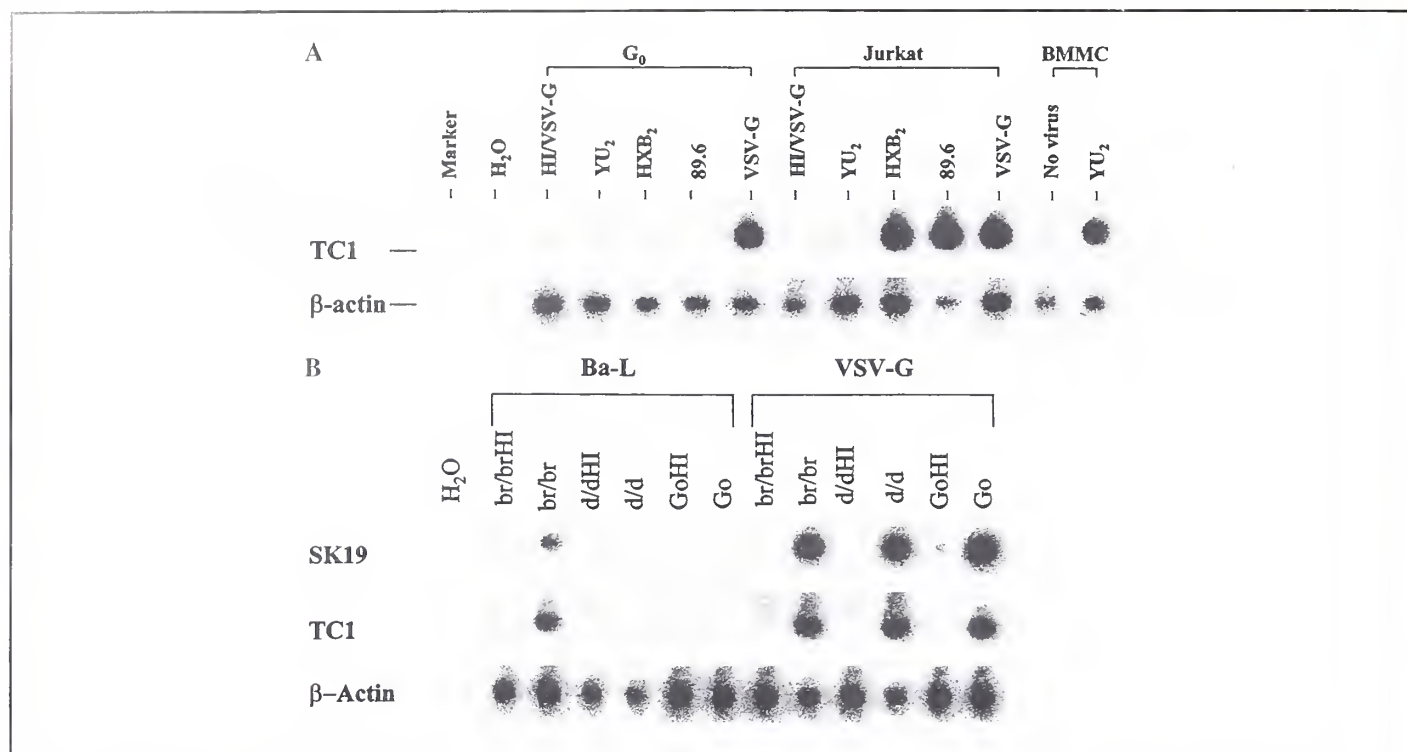


Fig. 5. Stem cell block to human immunodeficiency virus (HIV)-1 infection regardless of HIV-1 tropism is confirmed in stem cells derived by independent methods and can be circumvented by pseudotyping in VSV G enveloped virus. **A)** G₀ cells were exposed to recombinant HIV-1 pseudotyped in envelopes of M-tropic (YU₂), T-tropic (HXB₂), or dual tropic (89.6) specificity or in an envelope that contained VSV G. Cells were analyzed by DNA polymerase chain reaction (PCR) for early HIV transcripts. Heat-inactivated VSV G pseudotyped virus (HI/VSV-G) was used to control for virus infectivity. Jurkat and BMMC cells were used as positive control cell lines to control for HIV-1-enveloped virus infectivity. Lambda DNA (marker) or water alone (H₂O) were used as PCR

controls. **B)** Cells isolated by either Rhodamine/Hoechst staining with bright/bright (br/br), representing more differentiated cells, and dim/dim (d/d), representing stem cells, or by cytokine nonresponsiveness (G₀) were exposed to wild-type, Ba-L, or pseudotyped HIV within a VSV G envelope and evaluated for infection by PCR directed against late (SK19) or early (TC1) reverse transcription products. HI designates heat-inactivated control virus preparations. All products were confirmed by radiolabeled specific oligonucleotide hybridization as shown after phosphorimager analysis. Figure 5. B reprinted with permission by the American Society for Microbiology.

STEM CELLS AS THERAPEUTIC TOOLS

The data presented here indicate that the hematopoietic stem cell is resistant to HIV-1 infection *in vitro* and *in vivo* despite receptor and co-receptor expression. The stem cell is, therefore, not a potential long-lived reservoir of virus and is an appropriate cell to consider in autologous gene therapy approaches to acquired immunodeficiency syndrome (AIDS). To the extent that this cell can be recovered from AIDS patients, it may be envisioned to be a virus-free cell type that may be transduced with anti-HIV constructs for possible immune reconstitution. The ability to transduce the stem cell with HIV-based constructs was also documented in this study but was restricted to constructs pseudotyped in VSV G. There appears to be no block to such constructs entering quiescent stem cells and, at least transiently, expressing a transgene. Integration of the transgene or durable expression was not tested in this study, but another report (32) has indicated that HIV-based vectors may indeed result in sustained transgene expression in transplanted hematopoietic cells. Thus, stem cells may ultimately be manipulated to serve as a component of therapies of the future designed to replace the damaged immune system of HIV-1-infected individuals.

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NOTES

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Regulation of Bcl2 Phosphorylation and Potential Significance for Leukemic Cell Chemoresistance

Xingming Deng, Steven M. Kornblau, Peter P. Ruvolo, W. Stratford May, Jr.

Although considered tightly linked, the linkage effectors for proliferation and antiapoptotic signaling pathways are not clear. Phosphorylation of Bcl2 at serine 70 is required for suppression of apoptosis in interleukin 3 (IL-3)-dependent myeloid cells deprived of IL-3 or treated with antileukemic drugs and can result from agonist activation of mitochondrial protein kinase C α (PKC α). However, we have recently found that high concentrations of staurosporine up to 1 μ M can only partially inhibit IL-3-stimulated Bcl2 phosphorylation but completely block PKC α -mediated Bcl2 phosphorylation *in vitro*, indicating the existence of a non-PKC, staurosporine-resistant Bcl2 kinase (SRK). Although the RAF-1-MEK-1-mitogen-activated protein kinase (MAPK) cascade is required for factor-dependent mitogenic signaling, a direct role in antiapoptosis signaling is not clear. In particular, the role of phosphorylation in the regulation of death substrates is not yet clear. Our findings indicate a potential role for the MEK/MAPK pathway in addition to PKC in antiapoptosis signaling, involving Bcl2 phosphorylation that features a role for extracellular signal-regulated kinase (ERK)1 and 2 as SRKs. These findings indicate a novel role for ERK1 and 2 as molecular links between proliferative and survival signaling and may, at least in part, explain the apparent paradox by which Bcl2 may suppress staurosporine-induced apoptosis. Although the effect of phosphorylation on Bcl2 function is not clear, effector molecules that regulate Bcl2 phosphorylation may have clinical significance in patients with acute myelogenous leukemia (AML) who express detectable levels of Bcl2. Preliminary findings suggest that expression of PKC α , ERK2, and Bax in leukemic blast cells from patients with AML, although individually not prognostic, appears to have potential clinical value in predicting chemoresistance and survival outcomes. [J Natl Cancer Inst Monogr 2000; 28:30-7]

Hematopoietic growth factors, such as interleukin 3 (IL-3), mediate cell growth by stimulating proliferation and by suppressing the process of programmed cell death (1-4). A great deal is known about the molecular components and mechanisms that regulate IL-3 and other cytokine superfamily receptor-mediated signal transduction pathways that result from receptor dimerization and activation of nonreceptor protein tyrosine kinases like JAK2, with coupling to the activation of cytoplasmic signal transducers and activators of transcription (3,5,6) or the activation of the Src-homology collagen, growth factor receptor-bound protein 2, son of sevenless-coupled RAS/RAF-MEK-1/mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway (3,7-9). However, relatively little is understood about the postreceptor signaling mechanism(s) by which growth factors, such as IL-3, might also couple to and regulate apoptosis.

Bcl2 and related family members are key regulators of pro-

grammed cell death or apoptosis, a natural process required for normal development, and they play a role in malignant transformation and autoimmune diseases (10-14). Bcl2 was discovered in the oncogene hunt as the oncogene fusion product of the immunoglobulin H (IgH) promoter and full-length Bcl2 characterized by the t(14;18) breakpoint translocation found in approximately 80% of patients with indolent non-Hodgkin's lymphoma (13-15). The survival function of Bcl2 as a potent suppressor of apoptosis was initially demonstrated when Bcl2 was shown to facilitate prolonged survival following exogenous expression in IL-3-dependent hematopoietic cells that were deprived of IL-3 (16) and later through studies with transgenic and knockout mice (17-20). Thus, in the absence of IL-3, cells default to a suicidal apoptotic pathway involving intracellular proteolysis, which, in turn, can be inhibited by Bcl2 (16). Subsequently, Bcl2's ability to promote prolonged, but not indefinite, cell survival under various types of apoptotic stress (e.g., treatment with chemotherapy drugs, irradiation, exposure to toxins, or viral infection) was also discovered (16-18,21,22).

Work in our laboratory has uncovered a novel regulatory role for IL-3 in post-translational regulation of induced Bcl2 phosphorylation (23,24). The mechanism(s) by which IL-3 and other survival agonists may induce Bcl2 phosphorylation and the potential regulatory role for this post-translational modification on Bcl2's function will be the focus of this study.

BCL2 FUNCTIONS AS A DOCKING PROTEIN WITH POTENTIAL PORE-FORMING PROPERTIES

The Bcl2 family, which now numbers some 16 members, is made up of both suppressors (Bcl2, BCL_{XL}, and MCL-1) and inducers (Bax, Bad, Bak, and Bid) of apoptosis [reviewed in (10-12)]. Briefly, Bcl2 has four conserved Bcl2 homology (BH) functional domains and seven α -helical regions providing structure. The BH1, BH2, and BH3 domains are also contained in some pro-apoptotic death effector members, and mutational studies have shown these domains to be necessary for Bcl2-Bax heterodimerization and any potential Bcl2 or Bax pore-forming properties (25-29). The heterodimerization of Bcl2 and Bax recently has been formally demonstrated *in vivo* (30) and is currently considered important, at least in part, for Bcl2's ability to block Bax's potent death effector properties (25,30). On the basis of the recent crystallographic and solution structure of Bcl-X_L (31) and a BH3-only Bax-derived peptide, the BH3 domains in Bcl2, Bax, and other BH3-only members, such as Bid

Affiliations of authors: X. Deng, P. P. Ruvolo, W. S. May, Jr., University of Florida Shands Cancer Center, Gainesville; S. M. Kornblau, The University of Texas M. D. Anderson Cancer Center, Houston.

Correspondence to: W. Stratford May, Jr., M.D., Ph.D., University of Florida Shands Cancer Center, Box 100232, Gainesville, FL 32610-0232 (e-mail: smay@ufsec.ufl.edu).

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and Bim, are also modeled as death agonists (27,32,33). One popular model features their death-inducing effects occurring as a result of their potential membrane pore-forming properties that are potentially exerted as a result of binding to Bcl2 or Bcl-X_L and/or integrating into mitochondrial membranes (25,30,34). For example, Bax and the BH3-only Bid death effectors can bind to Bcl2 and become associated with the mitochondrial membranes. Apparently, when their death properties are revealed, they become integrally associated with mitochondrial membranes that potentially open megapore channel(s) (35). This presumably is accompanied by migration of Bax to the mitochondria in association with leakage of caspase activators to the cytosol and collapse of the mitochondrial membrane potential (28–30). In support of this model, the crystal structure of Bcl-X_L and Bax predicts a similar structure with that of bacterial colicins and diphtheria pore-forming toxins that function by disrupting membrane function (31,34–36). Furthermore, purified Bax can apparently directly induce mitochondrial membrane leakage in intact mitochondria *in vitro* by a process that can be blocked by Bcl2 (36). Thus, Bcl2 may function, at least in part, by docking with and/or somehow “neutralizing” Bax’s pore-forming properties (25,34–36). Alternatively, other functional properties of Bcl2 may result from its potential role as a multidocking site for other death regulators or components of mitogenic signal transduction pathways, including protein kinases (e.g., RAF-1) and phosphatases (e.g., protein phosphatase 2B [PP2B]) (Fig. 1)

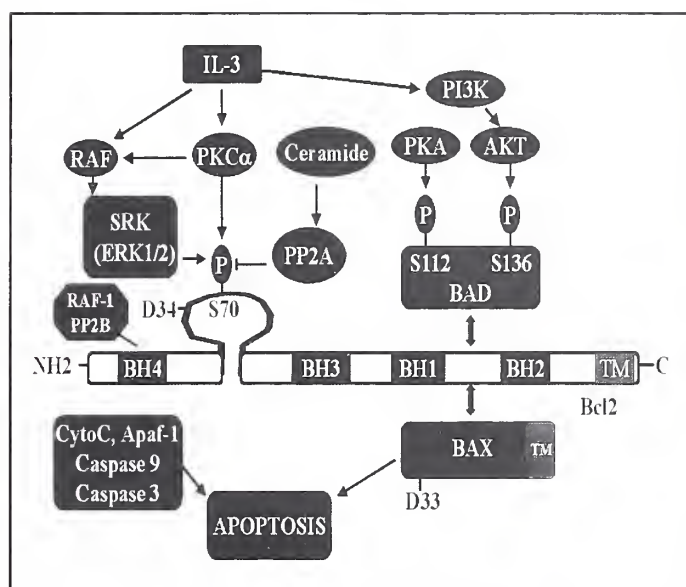


Fig. 1. Regulation of Bcl2 phosphorylation by interleukin 3 (IL-3) in factor-dependent myeloid cells. IL-3 activates two signal transduction pathways that can lead to Bcl2 phosphorylation at serine 70 by protein kinase C α (PKC α) and the RAF/MAPK/ERK-1, 2 (SRKs). PKC α can directly phosphorylate Bcl2 or indirectly through the activation of the SRKs ERK1 and ERK2 that also phosphorylate Bcl2 at serine 70. Although postreceptor coupling of IL-3 can activate the well-described RAS/RAF/MAPK/ERK1/2 pathway leading to direct phosphorylation of Bcl2 by MAPKs, ERK1, and ERK2, this pathway can also be activated by PKC α via a direct effect on RAF-1 (90). Bcl2 phosphorylation at serine 70 occurs within the flexible loop (aa 29–80) that is between the BH4 domain and the BH3 domain. The protein phosphatase PP2A can dephosphorylate Bcl2, and C2-ceramide can stimulate dephosphorylation. Bcl2 can heterodimerize with Bax or Bad, depending on the phosphorylation status of Bad affected by PI3-K or PKA. Bcl2 can regulate caspase 3 activation by regulating cytochrome C (CytoC) and apoptosis activation factor-1 (Apaf-1) release from the mitochondria. Also shown is the Bcl2 aspartate34 (D34) site of caspase 3 cleavage and the putative Bax cleavage site, aspartate33 (D33).

(37–39). Furthermore, in addition to Bcl2’s ability to act as a docking or scaffold assembly protein, it may alter the susceptibility of certain bound proteins to undergo posttranslational modifications, including proteolytic cleavage or phosphorylation that may potentially regulate their function. For example, Bcl2 can be inactivated following proteolytic cleavage (40,41). How or whether Bcl2 can regulate these processes is not yet clear, but our preliminary findings suggest that the phosphorylation state of Bcl2 may play a role in proteolytic cleavage of both Bcl2 and Bax. These new findings will be discussed below. In addition, Bcl2 and Bcl-X_L may also be regulated at the transcription level (42).

BCL2 IS FUNCTIONALLY PHOSPHORYLATED ON SERINE 70 BY IL-3 AND OTHER SURVIVAL AGONISTS

Bcl2 was initially identified as a potential phosphoprotein when expressed in SF9 cells in which it was shown to prolong cell survival following baculovirus infection (43). Later studies in our laboratory (23,24) discovered that IL-3 could induce a rapid and robust serine phosphorylation of Bcl2. Importantly, this modification correlated closely with cell survival in factor-dependent cells and suggested a functional role for phosphorylation. It is interesting that the potent protein kinase C (PKC) agonist and natural product bryostatin-1 (Bryo), which can also support survival of IL-3-dependent myeloid cells following IL-3 withdrawal, were found to induce Bcl2 phosphorylation, which initially suggested a functional role for PKC (23,24,44). Phosphorylation of Bcl2 was found to occur at the same serine site, whether induced by Bryo, IL-3, or the related erythroid hematopoietic hormone erythropoietin (23,24). Bcl2 mutational studies confirmed a functional role for phosphorylation at the evolutionarily conserved ser70 site, which is located in a putative regulatory region known as the flexible loop domain (FLD) (30). Thus, only Bcl2 containing the serine 70 to alanine (S70A) mutation failed to undergo phosphorylation by either IL-3 or Bryo, and this mutant also displayed a severely reduced survival function when stably expressed in factor-dependent cells (23,24). However, cells expressing the S70A Bcl2 mutant did fare slightly better with respect to survival than vector-only transduced parental cells, indicating that the nonphosphorylatable S70A Bcl2 mutant does retain some function under these conditions (24). This argues that phosphorylation may not be the only regulatory mechanism for Bcl2. In light of its multidocking and putative pore-channel properties, this is not surprising. By contrast, conversion of serine 70 to glutamate (S70E), a charged amino acid that could potentially mimic a phosphorylation site, resulted in an increased survival function. Thus, cells expressing S70E Bcl2 were more viable following the stress of either IL-3 withdrawal or etoposide chemotherapy treatment than cells expressing similar amounts of exogenous wild-type (wt) Bcl2 (24). These data strongly argue that ser70 is a regulatory site for Bcl2 (24) and allowed us to conclude that phosphorylation is necessary for Bcl2’s full and potent survival phenotype, at least in factor-dependent myeloid cells. Presumably, this extends to other growth factor-sensitive cells expressing Bcl2 because nerve growth factor (NGF) also induces Bcl2 phosphorylation in PC12 pheochromocytoma cells in association with survival (45). Of interest, dephosphorylation of Bcl2, even in the presence of NGF, is closely linked to apoptosis in these cells.

The serine 70 site of Bcl2 is evolutionarily conserved and is located within the predicted unstructured FLD of Bcl2 (31,46).

The FLD is a stretch of approximately 50 amino acids (aa 30–80) that resides between the putative $\alpha 1$ and $\alpha 2$ helical structures that separate the amino terminal BH4 and BH3 domains of Bcl2 (Fig. 1) (26,27,46). The potential loop domain is conserved between Bcl2 and Bcl-X_L, suggesting functional significance (46). It is interesting that deletion of this loop region from either Bcl-X_L or Bcl2 results in a molecule with enhanced survival function under specific circumstances, such as when expressed in WEHI-231 cells that undergo apoptosis following exposure to IgM (46,47). It has, therefore, been proposed that the FLD may represent a negative regulatory region (46). However, one report (48) that uses the identical Bcl2 loop deletion mutant has found that this domain is required for its survival function, at least when cells are treated with certain chemotherapeutic agents, including paclitaxel (Taxol). One explanation for this apparent paradox is that deletion of the large loop domain may functionally represent a “phosphorylation equivalent” mutation. Thus, if the FLD region were a negative regulatory region, phosphorylation might somehow “inactivate” its negative effect on survival. This possibility would be consistent with most reported findings in IL-3-dependent cells because, in the absence of IL-3 or a survival agonist, Bcl2 phosphorylation is not easily detected and the negative regulatory properties of the FLD may then dominate (24). Also consistent with this notion, forced overexpression of a nonphosphorylatable Bcl2 mutant (S70A) was unable to prolong cell survival following IL-3 deprivation or treatment with etoposide chemotherapy compared with wt or S70E Bcl2 (24).

BCL2 IS A SUBSTRATE FOR AT LEAST TWO BCL2 KINASES: PKC α AND A STAUROSPORINE-RESISTANT BCL2 KINASE

We have previously reported that PKC α is a physiologic Bcl2 kinase (44). However, the existence of another, non-PKC Bcl2 kinase(s) was also indicated, as overexpression of exogenous Bcl2 is reported to protect cells from apoptosis that would normally be induced by high concentrations of the potent PKC inhibitor staurosporine (49). Thus, a staurosporine-resistant Bcl2 kinase(s) (SRK) was sought. Involvement of an MAP kinase (i.e., ERK1 or ERK2) was considered likely according to reports that activation of the MAP kinase phosphatase-1 (MKP-1) was associated with Bcl2 dephosphorylation and apoptosis in NGF-dependent PC12 cells treated with angiotensin 2 (45). Because IL-3 can rapidly activate the RAF-MEK-1-MAPK pathway (50), we tested a role for an MAPK in Bcl2 phosphorylation. Preliminary studies that used various protein kinase inhibitors indicated that PD98059, a specific MEK-1 inhibitor, could, like staurosporine, only partially block IL-3-induced Bcl2 phosphorylation. However, the combination of PD98059 and staurosporine could completely shut down IL-3-induced Bcl2 phosphorylation (51). Thus, ERK1 (p44) and ERK2 (p42) were identified as potential candidate Bcl2 kinases. It is interesting that a distinct population of cytosolic ERKs was found to be located in the heavy membrane mitochondrial subcellular fraction, indicating potential as direct Bcl2 kinases. When individually tested, ERK1 and ERK2 were found to be potent, direct Bcl2 kinases (Fig. 1) (51). Collectively then, although these findings identify ERK1 and ERK2 as physiologic Bcl2 kinases, they cannot exclude the formal possibility that other SRKs may also exist (Fig. 1) (51).

BCL2 PHOSPHORYLATION IS A DYNAMIC PROCESS INVOLVING PHYSIOLOGIC BCL2 KINASE(S) AND A PHOSPHATASE

Although Bcl2's survival function can be regulated, at least in part, by phosphorylation at ser70, phosphorylation is not a static process (52). Rather, Bcl2 phosphorylation represents a balance between a Bcl2 kinase(s) and a phosphatase(s) (Fig. 1). Thus, the potential for perturbing Bcl2's survival function through agonist-induced Bcl2 phosphorylation may also occur via phosphatase activation (52). Concerning this possibility, it is interesting to note that ceramide, a potent PP2A activator (53,54), can be rapidly generated intracellularly after treatment of cells with various types of cell death stimuli, including cytotoxic cytokines like tumor necrosis factor α (55), chemotherapeutic drugs (56,57), ischemia/reperfusion injury (58), FAS antigen activation (59), irradiation (60), and corticosteroids (61). Indeed, the production of ceramide is so common during apoptosis that it has been considered a universal feature of this process (62,63). Whether ceramide is a trigger for cell death is not clear, but C2-ceramide can induce cell death when added directly to cells (55). We have discovered that C2-ceramide, but not the functionally inactive C2-dihydro-ceramide, can potently inhibit Bcl2 phosphorylation induced by either IL-3 or Bryo (64). Reversal of phosphorylation resulted from the rapid activation of a mitochondrial-associated, okadaic acid-sensitive PP2A-like activity that was directly associated with Bcl2. Of interest, however, cells expressing the functionally potent S70E Bcl2 mutant fail to undergo apoptosis after treatment with high concentrations of C2-ceramide that can potently activate PP2A and would readily induce apoptosis in cells expressing wt or S70A Bcl2 (64). These findings indicate that inhibition of Bcl2 phosphorylation may be one mechanism by which C2-ceramide can induce apoptosis in IL-3-dependent myeloid cells that express Bcl2. In support of this possibility, it was demonstrated that, although NGF can induce Bcl2 phosphorylation and survival in PC12W pheochromocytoma cells, NGF-induced Bcl2 phosphorylation could be inhibited and cells induced to undergo apoptosis after addition of angiotensin-2 (45). Angiotensin-2 was found to potentially activate the MAPK-phosphatase, MKP-1, that resulted in apparent inhibition of MAPK/ERK activity and was associated with loss of phosphorylation of Bcl2 (45). These findings are consistent with the notion that inhibition of Bcl2 phosphorylation is associated with apoptosis. However, because protein phosphatases and kinases seldom have a solitary substrate, it may be possible that the phosphorylation of other potential molecule(s) may affect the survival status of the cell. Furthermore, other potential Bcl2 kinases and phosphatases may also exist.

It has been reported that Bcl2 may bind and sequester the protein phosphatase PP2B in association with protection of Jurkat T cells from apoptosis induced by PP2B/calcinurin overexpression (39). Thus, although PP2B could be a potential Bcl2 phosphatase on this basis, we found that, at least *in vitro*, PP2B is a much weaker Bcl2 phosphatase than PP2A or PP1 (52). This finding suggests that PP2B's role in Bcl2's binding may not have a direct effect on phosphorylation and function; alternatively, Bcl2 may regulate PP2B's role in FAS-ligand-induced apoptosis of T cells by actively soaking up PP2B (65). Alternatively, because PP2B is, like BAD, located primarily in the cytosol, one other consequence of PP2B binding to Bcl2 may be to sequester this enzyme and to prevent dephosphorylation of

cytosolic substrates such as BAD, which can help trigger apoptosis under some circumstances (66–68).

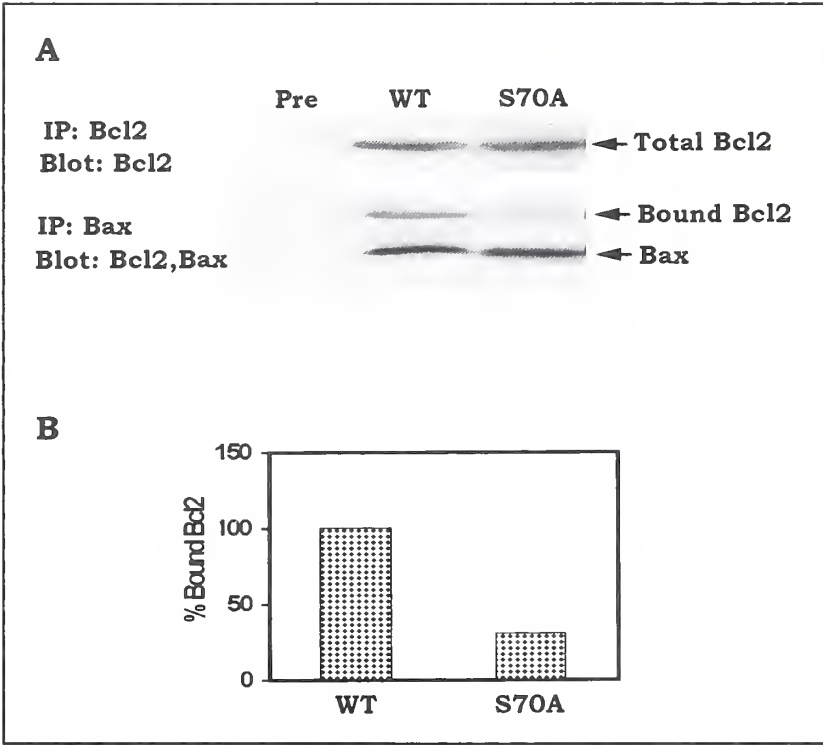
BCL2 PHOSPHORYLATION MAY POTENTIALLY AFFECT THE PROTEOLYTIC CLEAVAGE OF BOTH BCL2 AND BAX

It was reported that the N-terminal domain of Bcl2 could be proteolytically cleaved at a recognized caspase 3 proteolytic site at D34 (40). Furthermore, cleavage of Bcl2 renders a truncated form (Δ 34N-Bcl2) that is nonfunctional in protecting cells from IL-3 deprivation. These data suggest that the cleaved N-terminal region of Bcl2, which contains the BH4 domain that is the docking site for such signaling proteins as RAF-1, PP2B, and p53 BP2, is potentially required for its potent antiapoptotic activity. Thus, IL-3 postreceptor signaling may somehow protect Bcl2 from inactivation by caspase cleavage (40). Our preliminary findings (24) support this notion. We found that steady-state expression of Bcl2 is maintained and cell survival prolonged after IL-3 deprivation in cells that express wt but not S70A-Bcl2 (Fig. 2). This finding suggests that the ser70 site phosphorylation of Bcl2, although not being required for Bax heterodimerization, may protect it from proteolytic cleavage. Precisely how phosphorylation may affect this process is not yet clear. It is interesting that phosphorylation can apparently protect procaspase 9 and presenilin-2 from proteolytic cleavage (69,70). Presenilin-2 is a transmembrane protein potentially involved in early onset of Alzheimer's disease (70). Phosphorylation at a serine site residing c-terminal to an aspartate target site for caspase apparently retards the neuronal apoptotic process characteristic of this neurodegenerative disease (70).

Although Bcl2 is an integral mitochondrial membrane protein that heterodimerizes with Bax, the majority of Bax is not an integral membrane protein, at least during normal cell growth (71,72). Rather, Bax is primarily cytosolic and/or only periph-

erally associated (i.e., not membrane integrated) with the mitochondria membranes (such that it can fractionate with mitochondria unless extracted by a pH 11.5 alkali treatment to remove peripherally associated proteins) (71). Bax can be translocated during stress from the cytosol to the outer mitochondrial membrane, where it will apparently integrate into the membrane via its hydrophobic c-terminal transmembrane domain (71–73). However, how Bax is cleaved and/or translocated from the cytosol to become an integral membrane protein that may trigger or be involved in apoptosis is not yet clear. Bax is a 21-kd protein. It was found that p21 Bax can be cleaved at the N-terminus to yield a p18 Bax form that apparently is more efficient at membrane insertion, at least *in vitro* (71). Our preliminary data suggest that Bcl2 phosphorylation may enhance, at least in part, the stability of the interaction between Bcl2 and Bax (25) and potentially retard Bax cleavage. Our findings also indicate that an intact Bcl2 ser70 phosphorylation site is required to maintain the tight association between Bax and Bcl2 observed during co-immunoprecipitation from detergent lysates of cells (Fig. 2). Thus, the nonphosphorylatable, severe loss of function of S70A Bcl2 displays a significantly decreased association with Bax. Although Bcl2 phosphorylation is not required for Bax: Bcl2 association, it may stabilize such an association. Indeed, ceramide-induced Bcl2 dephosphorylation also appears to correlate with a similar decrease in Bcl2: Bax association (Fig. 3). Importantly, cells expressing the S70E Bcl2 mutant, which mimic phosphorylation but cannot be dephosphorylated, are viable even at elevated concentrations of ceramide (i.e., 50 μ M). By contrast, cells expressing wt Bcl2 are killed at ceramide levels (10 μ M) in which serine 70 is dephosphorylated (64). Furthermore, under conditions of IL-3 deprivation, Bax undergoes rapid proteolytic cleavage from a p21 to a p18 Bax form (Deng X, Ruvolo P, Carr BK, May WS: unpublished data). Thus, Bax cleavage is pronounced and occurs in cells that express S70A Bcl2 after IL-3 withdrawal. This finding suggests

Fig. 2. Bcl2 phosphorylation at ser70 is necessary for maximal and stable association with Bax. **A)** NSFN1/H7 cells were stably transfected with wild-type (wt) or S70A mutant Bcl2 and grown in interleukin 3 (IL-3)-containing media. Cells were harvested, washed, and lysed in detergent buffer. The lysates were then immunoprecipitated with the use of a Bax antisera as described previously (23). The immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel, transferred to a PVDF membrane, and simultaneously analyzed by western blot analysis with a mixture of Bcl2 and Bax antisera (**lower panels**). Total cell Bcl2 was determined by quantitative immunoprecipitation of Bcl2 from the cell lysate with the use of a Bcl2 antisera (**upper panels**). **B)** Bax-associated wt and S70A Bcl2 is calculated with the use of densitometry analysis and expressed as a percentage of the bound Bcl2.



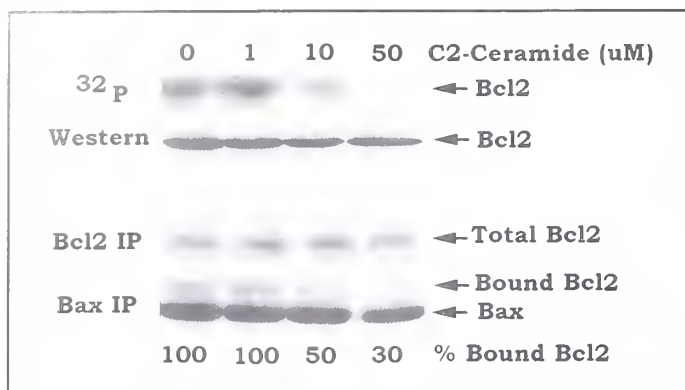


Fig. 3. C2-ceramide addition to cells induces Bcl2 dephosphorylation and is associated with a decrease in Bcl2 : Bax association. Ceramide has been demonstrated to promote the dephosphorylation of wild-type (wt) Bcl2 (64). Co-immunoprecipitation of wt Bcl2 : Bax. Bcl2 : Bax is reduced in a dose-dependent manner, following addition of C2-ceramide to cells before detergent lysis and processing as described in the legend to Fig. 2. Bcl2 : Bax ratio is calculated with the use of densitometry analysis and is expressed as a percentage of the bound Bcl2 : Bax in the absence (100%) and following addition of C2-ceramide at concentrations of 1, 10, and 50 μ M.

that phosphorylation of Bcl2 at ser70 can modulate Bcl2 : Bax stability and potentially protect p21 Bax from proteolysis. How Bcl2 phosphorylation may protect Bax and/or Bcl2 itself from proteolysis and whether cleavage of these substrates may be involved in initiating and/or amplifying the process as compared with simply being degraded during apoptosis are not yet clear.

In addition to Bcl2 and Bax, pro-caspase 9, an initiator of the intrinsically activated caspase cascade (74,75), is also apparently regulated by phosphorylation and proteolytic cleavage (69). Thus, following AKT-induced pro-caspase 9 phosphorylation at ser196, the pro-caspase form remains intact and catalytically inactive (67). A flag-tagged ser196 ala pro-caspase 9 mutant was created that was found to be resistant to AKT-mediated phosphorylation and, importantly, unable to undergo caspase 3 cleavage and enzymatic activation. Thus, post-translational phosphorylation mechanisms may be commonly employed in the regulation of cleavage substrates in the apoptotic pathway. Our finding that phosphorylation of Bcl2 at ser70 is required for its full and potent survival function may potentially be explained by a role in regulating cleavage of itself and/or its heterodimeric, pro-apoptotic partner Bax (Fig. 2).

MULTISITE BCL2 PHOSPHORYLATION

In addition to our previous findings (23,24,44) and those of others (76–78) concerning a role for phosphorylation in regulating Bcl2's survival function, serine phosphorylation of Bcl2 has also been reported to result from the treatment of cells with specific antimitotic chemotherapeutic agents, including paclitaxel, vincristine, vinblastine, and dolastatin 10 (79–84). Because cells undergo apoptosis after exposure to these toxins, it was proposed that phosphorylation could negatively regulate or inactivate Bcl2 (79). However, drug-induced Bcl2 phosphorylation is markedly different from that seen after the addition of growth factors or other survival agonists. First, although IL-3-induced Bcl2 phosphorylation is rapid and occurs within minutes, paclitaxel induces a slow phosphorylation (i.e., 2 hours) that occurs during mitosis only (79–84). Second, unlike IL-3- or NGF-induced Bcl2 phosphorylation (23,24,44,45), paclitaxel-

induced phosphorylation is associated with a nonreversible or slowly reversible mobility shifted form of Bcl2 detected by western blot analysis following denaturing electrophoresis (77–84). Third, the Bcl2 kinases responsible for this drug-induced phosphorylation mechanism are reported to be protein kinase A (PKA) and c-Jun N-terminal kinase (JNK) (76,82,83). PKC α and ERK1 or ERK2 are apparently not involved. Furthermore, paclitaxel-induced Bcl2 phosphorylation apparently occurs at three sites, including thr69, ser70, and ser87 (81,83), and Rac1-activated JNK was found to phosphorylate Bcl2 directly *in vitro* at multiple sites, including thr56, thr74, ser70, and ser87 (76). Thus, it is possible that mono-site (i.e., ser70) versus multiple-site Bcl2 phosphorylation may differentially affect Bcl2 function, perhaps by inducing different conformational changes in the molecule. However, although antimitotic drug treatment is associated with cell death, the cells expressing the nonshifted and unphosphorylated Bcl2 form are apparently the ones that actually undergo apoptosis (85). Thus, apoptosis likely occurs from the well-characterized mechanism by which such drugs deregulate the dynamic microtubule function (86), and Bcl2 phosphorylation may not be required or involved. To date, it has not been experimentally demonstrated that multisite phosphorylation of Bcl2 renders Bcl2 functionally inactive to suppress apoptosis. Such conclusions have been largely based on circumstantial data. An alternative explanation not yet tested is whether multisite Bcl2 phosphorylation might represent an unsuccessful attempt by the cell to activate and engage any survival mechanism(s) available, but, as a result of the irreversible microtubule damage sustained, cell death is inevitable. This alternate possibility predicts that cells expressing wt Bcl2 will display prolonged cell survival versus nonexpressing cells when treated with antimitotic drugs even if the cells eventually undergo apoptosis. Furthermore, expression of Bcl2 mutants containing a double mutation at both ser70 and ser87 sites to nonphosphorylatable amino acids would be predicted to inhibit apoptosis after paclitaxel treatment. Although there is no evidence available yet that tests the latter prediction, it has been reported that expression of wt Bcl2 can significantly prolong cell survival after exposure to paclitaxel (87). These findings then appear to indicate that treatment with antimitotic agents does not block Bcl2's antiapoptotic function but rather that Bcl2 can protect against such drug-induced death. Further studies will be required to test the effect of multisite Bcl2 phosphorylation on survival function.

EXPRESSION OF PKC α , BAX, AND ERK1 AND ERK2 IN CLINICAL AML SAMPLES MAY MODULATE BCL2'S PROGNOSTIC SIGNIFICANCE FOR PATIENT OUTCOMES

On the basis of the above findings and our preliminary studies, we have tested whether expression of the Bcl2 kinase PKC α and Bcl2 and Bax may have clinical relevance. Earlier, we reported that increased expression of the Bcl2 protein in patient samples of AML cells displaying favorable or intermediate prognosis cytogenetics (FIPC) correlated with decreased rates of successful remission-induction treatment and event-free survival (88). Samples of leukemic blast cells from 165 patients with newly diagnosed AML were obtained from peripheral blood samples (approximately 85% blasts) and analyzed as individual and interactive variables (89). When assessed individually, the expression levels of PKC α or Bax, as compared with Bcl2 (89), were not prognostic of successful standard induction-remission or survival outcomes. However, when evaluated as interactive

Table 1. Effect of BAX, PKC α , Bcl2/BAX, and PKC α · Bcl2/Bax ratios in response to therapy and survival in patients with FIPC AML*

Protein	Remission rate < median, %	Remission rate > median, %	P (Fisher's exact test)	Survival < median, wk	Survival > median, wk	P†
Bax	72	87	.12	89	132.5	.08
PKC α	82	77	.61	124	92.5	.48
Bcl2/Bax	88	69	.04	141	80.5	.007
PKC α /Bcl2	82	77	.60	132	88	.38
PKC α · Bcl2/Bax	92	65	.002	141.5	55	.005

*From October 1991 through July 1995, 100 patients with newly diagnosed, untreated acute myelogenous leukemia (AML) with favorable or intermediate cytogenetics were evaluated at The University of Texas M. D. Anderson Cancer Center, Houston. Samples for this study were acquired during routine diagnostic assessment in accordance with regulations and protocols sanctioned by the Human Subjects Committee of The University of Texas M. D. Anderson Cancer Center. A Ficoll-generated mononuclear fraction of peripheral blood was obtained for analysis. Median protein levels were scored by densitometry from western blot analysis from patient samples and normalized against control signals from K562 or Y79 cells. PKC α = protein kinase C α ; FIPC = favorable or intermediate prognosis cytogenetics.

†Gehan-Wilcoxon test.

variables, we found that the ratio of either Bcl2 to Bax (B2/Bx) or PKC α · B2/Bx (PK · B2/Bx; i.e., ratios of expression levels of the protein relative to the median level of expression of the individual protein) was highly prognostic for 100 patients with AML who exhibited FIPC (Table 1) (89). Results indicate that the AML samples that displayed a lower ratio of either B2/Bx or PK α · B2/Bx had a significantly higher initial remission-induction rate (88% versus 69%; $P = .04$) and a prolonged survival (median 141 weeks versus 80.5 weeks, $P = .007$) compared with patients whose blasts demonstrated higher ratios (89). Because a previous correlation was established for Bcl2 expression, a poor outcome but no correlation was observed in these preliminary studies to indicate that expression of individual levels of PKC α or Bax affected outcomes. The expectation was that, when forming the interactive variable terms (i.e., ratios), any prognostic value of Bcl2 alone would be lost (because of the expression of essentially random levels of PKC α or Bax). Surprisingly, however, forming the interactive terms gave greater prognostic discrimination, suggesting that, although the relationships were not immediately apparent on the basis of raw expression levels, a functional relationship among these variables exists. More recent preliminary studies have also suggested that expression of higher levels of ERK2 may also affect Bcl2's poor prognostic effect on AML (Kornblau SM, Ruvolo P, Deng X, May WS; unpublished data). A similar analysis of ERK1 as another Bcl2 kinase is now pending. No definitive conclusions should be drawn at this point from this retrospective analysis because the actual Bcl2 phosphorylation state and the apoptosis rate of individual AML leukemic blast cells were not measured. However, these results were found to be statistically significant and thus suggest that a functional relationship may exist between these variables. Further studies are now in progress to test the role for these variables in a prospective study. If a correlation can be established between cell survival and increased Bcl2 phosphorylation, mitochondrial localization of PKC α and/or ERK1 and ERK2, and increased cell survival following exposure to induction-remission chemotherapy *in vitro*, these data would support the hypothesis that phosphorylation of Bcl2 may have clinical relevance. In this case, developing novel antineoplastic strategies to block Bcl2 phosphorylation would be one novel strategy to improve both remission-induction success rates and survival for patients with AML.

In summary, it now seems clear that, in addition to a requisite role in growth factor-induced proliferative signaling, the MEK-1/MAPK (ERK1 and ERK2) pathway can functionally interface

with a survival signaling pathway induced by growth factors like IL-3 that feature Bcl2 phosphorylation. This finding now directly links these two critical pathways (Fig. 1). Furthermore, by serving as an SRK, the ERKs can potentially cooperate with other survival signaling pathways, including PKC activation, to ensure Bcl2 survival function. Our findings also help to explain the apparent paradox in which Bcl2 may remain functionally phosphorylated and at the same time protect cells from apoptosis induced by high concentrations of staurosporine, the potent inhibitor of PKC. Finally, if these findings are shown to have clinical relevance, this would suggest that novel apoptosis-inducing antineoplastic strategies aimed at functionally inactivating Bcl2 may require targeting of multiple agonist-activated upstream pathways.

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NOTE

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Post-transplant Lymphoproliferative Disorders: Implications for Acquired Immunodeficiency Syndrome-Associated Malignancies

Lode J. Swinnen

Post-transplant lymphoproliferative disorders (PTLDs) comprise a histologic spectrum, ranging from hyperplastic-appearing lesions to frank non-Hodgkin's lymphoma or multiple myeloma histology. Multiple clones may coexist, each representing a discrete lymphomagenic event, a situation that is unique to immunodeficiency states. The incidence varies from 1% in renal recipients to 5% in heart recipients, but can be markedly increased by the use of anti-T-cell therapies or by T-cell depletion in bone marrow transplantation. PTLD continues to arise, even many years after transplantation, and late T-cell lymphomas have recently been recognized. Pretransplant Epstein-Barr virus (EBV) seronegativity increases risk to as high as 30%–50%. PTLD has a highly variable clinical picture; certain patterns are, however, seen. Reversibility of PTLD with reduction in immunosuppressives has long been recognized. Predicting reversibility has been difficult. The presence or absence of *bcl-6* mutations has recently been identified as being of predictive value. Surgical resection can be curative. Cytotoxics, although problematic, can also be curative. Long-term remission has been achieved with anti CD21 and CD24 antibodies; efficacy has been reported for interferon alfa and for rituximab. *In vitro* expanded EBV-specific T cells have been effective as treatment and as prophylaxis in the setting of bone marrow transplantation. EBV viral load measured in blood appears to associate with the emergence of PTLD and may facilitate prophylactic studies. PTLD is a model of immunodeficiency-related EBV lymphomagenesis. Pathogenetic, therapeutic, and prophylactic insights gained from the study of PTLD are likely to be applicable to the acquired immunodeficiency syndrome setting. [J Natl Cancer Inst Monogr 2000;28:38–43]

Malignancy following iatrogenic immunosuppression has been recognized since the beginning of organ transplantation (1). More than 20 000 organ transplants are performed per year in the United States. Longer patient survival, the increasing use of organ transplantation in the pediatric age group, and the use of potent and highly T-cell-specific immunosuppressive agents all contribute to an increasing incidence of post-transplant malignancy (2–6).

IMMUNOSUPPRESSIVE REGIMENS

Immunosuppressive regimens vary from one institution to another, and a number of new agents has recently been introduced. Regimens are commonly based on an agent that inhibits T-cell function, such as cyclosporine or FK506, in combination with azathioprine and prednisone. Immunosuppression is most intense immediately after transplantation, with gradual reduction in dosage over the ensuing years. The incidence of post-transplant lymphoproliferative disorder (PTLD) is higher in non-

renal than in renal recipients. The most reliable estimates of incidence currently available are based on data obtained by the European and North American Collaborative Transplant Study. Those data are population based and multicenter and take length of follow-up into account. PTLD incidence among 45 141 renal recipients and 7634 cardiac recipients was determined. As had been noted in other series, incidence was highest in the first year after transplant. During that first year, 0.2% of renal and 1.2% of cardiac recipients developed PTLD, rates that were calculated to be 20 and 120 times higher than those seen in the general population. The incidence of PTLD in subsequent years was about 0.04% per year in renal and 0.3% per year in cardiac recipients (2). In a subsequent report, analyzing 14 284 heart recipients and 72 360 kidney recipients, a cumulative incidence of almost 5000 per 100 000 by 7 years of follow-up was noted in heart recipients and slightly more than 1000 per 100 000 by 10 years of follow-up in renal recipients, a cumulative incidence of about 5% for heart recipients and 1% for kidney recipients (3). A striking feature of this analysis is the fact that PTLD continues to arise even many years after transplantation, despite the fact that therapeutic immunosuppression is reduced over time. Prolonged survival in the face of immunodeficiency is, therefore, associated with a continued risk of lymphoid malignancy in the setting of organ transplantation. The effect of highly active antiretroviral therapy on the incidence of lymphoid malignancy in the setting of acquired immunodeficiency syndrome (AIDS) is as yet unclear. Despite improvement in CD4 counts and relatively good immune function on such therapy, the transplant experience raises some concern that the problem of lymphoma may not be obviated.

The risk of PTLD appears to be influenced by quantitative and qualitative differences in the degree of immunodeficiency. Statistically significantly higher doses of cyclosporin and azathioprine were found in heart than in kidney recipients on analysis of Collaborative Transplant Study data. A related observation was the significantly higher risk for PTLD among North American recipients than for patients transplanted at European centers, amounting to a relative risk of 2.12, which was associated with, and attributed to, higher immunosuppressive dosage among North American recipients (2). Highly potent anti-T-cell therapy has furthermore been shown to markedly influence the incidence in both organ and allogeneic bone marrow recipients. A ninefold higher incidence of PTLD was noted among patients who had received induction therapy with the immunosuppressive antibody OKT3 (11.4% versus 1.3%) in a series of cardiac transplant recipients (6). A strong dose response was observed, in that

Correspondence to: Lode J. Swinnen, M.D., Division of Hematology/Oncology, Loyola University Chicago, Cardinal Bernardin Cancer Center, Rm. 245, 2160 S. First Ave., Maywood, IL 60153 (e-mail: lswinne@luc.edu).

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6.2% of the patients who had received one course of the drug and 35.7% of the patients who had received two courses developed the disease. A murine monoclonal antibody directed against the human CD3 receptor-T-cell complex, OKT3 profoundly depletes circulating CD3⁺ T lymphocytes. Similar effects have been noted in other series (2,7,8) and with T-cell depletion of donor marrow to reduce graft-versus-host disease in allogeneic bone marrow transplantation. PTLT is relatively uncommon (<1%) in the absence of such manipulations (9), whereas incidences of 12%–24% have been reported after T-cell depletion (10,11). It is not clear whether a risk threshold can be defined in organ recipients by qualitative or quantitative measures of immune function, but such information would be of relevance to the long-term management of AIDS, now that at least partial immune reconstitution is feasible. In the AIDS setting, the risk of Epstein-Barr virus (EBV)-associated immunoblastic lymphoma, and particularly primary central nervous system (CNS) lymphoma, has been associated with severe reduction in CD4 counts (12), but it is likely that more subtle gradations of risk exist.

EBV ASSOCIATION

The malignancies most clearly over-represented in organ transplant recipients are squamous skin and cervical carcinomas, B-cell lymphomas, and Kaposi's sarcoma (13), all known to be virally related tumors. The majority of PTLTs have been EBV associated, with approximately 5% of tumors being clearly EBV negative. This situation is significantly different from that seen in the AIDS setting in which EBV-negative tumors make up a third or more of the total (14), a discrepancy with other immunodeficiency states that is as yet unexplained. The tumor-associated EBV is clonal in PTLT, supporting an etiologic role for the virus rather than for subsequent infection of neoplastic B cells. The pattern of viral clones furthermore corresponds to B-cell clonality as determined by immunoglobulin gene rearrangement analysis. Lesions consisting of polyclonal or multiclonal B-cell proliferations contain multiple EBV clones, whereas monoclonal proliferations show evidence of a single infectious event (15,16).

The incidence of PTLT is much higher in patients who are EBV seronegative prior to transplant (4,5). The risk of PTLT in EBV-seronegative recipients in one series was determined to be 76 times that in seropositive recipients (17). Most adults (90% or more) are EBV seropositive. The majority of seronegatives are children, with a higher likelihood of seronegativity the younger the child (4,18). A series from the University of Pittsburgh (PA) identified a four times higher risk of PTLT for pediatric than for adult transplant recipients (4). PTLT is, therefore, a particular problem among pediatric transplant recipients, and the risk has been considered to be sufficiently high as to preclude transplantation in some instances (17).

Analysis of archived liver biopsy specimens in liver transplant recipients has shown the presence of EBV, as determined by polymerase chain reaction (PCR) or by *in situ* immunohistochemical staining for EBER-expressing cells, in 70% of patients who subsequently developed PTLT. Only 10% of the patients who did not go on to develop the disease had such findings (19). Identification of a preclinical phase for PTLT would be highly desirable, in that early intervention might obviate the emergence of more aggressive proliferations. Viral load, as determined in peripheral blood mononuclear cells, has

been found to increase around the time of clinically detected disease (20,21). It is not clear whether such rises in EBV load indicate EBV-driven neoplasia or are simply reflective of severe immunodeficiency at the time. One extensive study (22) of EBV load in peripheral blood mononuclear cells addressing this question showed that rises in viral load occurred at some point in the post-transplant course of 73% of the patients studied. Although the mean rise in patients with PTLT was more than threefold higher, levels in patients who did not develop PTLT frequently equalled or exceeded the viral load seen in those who did. Viral load as determined in circulating mononuclear cells may, therefore, lack the specificity needed for a screening test. Attempts at defining quantitative standards with predictive value are under way, and a number of transplant centers have started monitoring patients with EBV viral load assays (23). Of interest, EBV DNA can be measured in serum from patients with PTLT with the use of very sensitive PCR techniques. In preliminary work, very high sensitivity and specificity for PTLT has been identified. It is not clear whether the presence of EBV DNA in serum might reflect tumor metabolism (24). Unlike oral hairy leukoplakia in immunodeficiency, PTLT has not been associated with a fully productive viral lytic cycle (25–27). Nevertheless, the viral Bzlf-1 protein, representing the initial entry into the lytic pathway, is generally expressed in PTLT, and there have been indications that at least partial virion production may occur (25–27).

The ability to monitor patients at risk for EBV-associated lymphoproliferations, and to intervene at a subclinical point in the disease process, is clearly attractive. PTLT represents a good model system for developing and testing such approaches. It may then be possible to apply a similar strategy to human immunodeficiency virus (HIV) disease, a setting in which HIV viral load monitoring has proven to be clinically feasible and highly useful in guiding therapy.

PATHOLOGY AND GENETIC ALTERATIONS

The term PTLT encompasses a range of lymphoproliferations, extending from reactive or hyperplastic-looking morphologies to a picture indistinguishable from immunoblastic non-Hodgkin's lymphoma (28–30). Several pathologic classification systems have been proposed (28,29,31). Correlative molecular genetic analysis has recently been included to allow grouping into three relatively distinct entities. 1) Plasmacytic hyperplasia is usually polyclonal, arising from the tonsils or cervical lymph nodes, containing multiple EBV infection events, and lacking oncogene or tumor suppressor gene alterations. It is not clear to what extent this entity differs from infectious mononucleosis in the setting of immunodeficiency. 2) Polymorphic B-cell hyperplasia and polymorphic B-cell lymphoma are usually monoclonal, containing a single form of EBV, and lacking oncogene and tumor suppressor gene alterations. 3) Immunoblastic lymphoma or multiple myeloma is monoclonal, containing a single form of EBV, and containing one or more structurally altered genes (N-RAS, C-MYC, p53, or others) (30). A recent Society of Hematopathology workshop on the subject recognized a number of other not clearly categorizable morphologies encountered among PTLTs (32).

Relatively few PTLT cases have been studied for cytogenetic abnormalities. These abnormalities have typically been seen in tumors with monomorphic histology, but no distinct abnormality has been identified as being characteristic for PTLT. In a recent series of 28 patients, no clonal cytogenetic abnormalities were

identified among 10 polymorphic tumors, all of which were polyclonal or oligoclonal. Analysis of 12 monomorphic cases revealed a variety of abnormalities in 10 cases: chromosome 8 translocations involving the MYC gene, trisomy 9, trisomy 11, and 11q27 (33).

The existence of more than one clone within the same lesion as well as both differing clones and differing histopathology in lesions at separate anatomic sites in the same patient is a characteristic feature of PTLD (34,35). Such multifocal clonal evolution contrasts sharply with the homogeneity seen in classic non-Hodgkin's lymphoma and likely represents a different mechanism for lymphoid neoplasia in immunodeficiency. Under suitable conditions, EBV-driven lymphoproliferation may appear simultaneously at multiple sites in the body. Similar findings have been described in AIDS-associated adenopathy and EBV-related tumors. EBV-negative tumors and small noncleaved histology are, however, infrequent in the PTLD setting. That difference is currently unexplained. It might relate to differences in the nature of immunodeficiency, to the state of immune activation that characterizes HIV disease, which has no counterpart in the organ transplant setting other than perhaps the presence of the allograft, or to other as yet unknown factors.

The vast majority of PTLDs studied have been of B-cell origin. EBV-negative aggressive T-cell lymphomas have been identified as a rare, very late occurrence, presenting at a median of 15 years after transplantation (36). Both EBV-associated and EBV-negative T-cell PTLD have been identified in other studies (32,37). The emergence of new entities, presumably immunodeficiency associated, at late time points after transplantation should be of some interest with regard to HIV disease, in which prolonged survival in the face of continuing immunodeficiency is now a realistic prospect.

CLINICAL ISSUES

The clinical presentation and behavior of PTLD are very heterogeneous. Extranodal disease and rapid tumor growth are frequently seen, as has been the case with the AIDS-related lymphomas. Despite the variability of PTLD, some clinical patterns have been identified. An infectious mononucleosis-like presentation, with prominent constitutional symptoms and rapid enlargement of the tonsils and cervical lymph nodes, is often the picture in the early post-transplant period—less than about 6 months to 1 year from the time of transplant (38,39). Highly immunosuppressed patients may present with widespread disease as well as diffusely infiltrative multiorgan involvement and pursue a fulminant clinical course (1,40). Lesions are usually polyclonal or oligoclonal in composition, and tumor histology is polymorphic rather than monomorphic. An analogous situation has not clearly been seen in the AIDS setting.

PTLD presenting later than about 1 year after transplantation tends to be more monomorphic, manifests fewer constitutional symptoms, and runs a more gradual clinical course. Clinically, this picture most closely resembles the AIDS-related immunoblastic or large-cell lymphomas. Gastrointestinal involvement is a frequent finding in PTLD (1). The transplanted organ itself appears to be a preferred site of involvement, being affected in up to 20% of cases. Central nervous system (CNS) involvement at presentation is mainly seen as part of very extensive disease. A clinical picture resembling myeloma can occur, with lytic bone lesions or the production of a monoclonal paraprotein (40,41).

A number of different approaches to treatment has met with a degree of success. PTLD is nonetheless a rapidly progressive, frequently lethal disease. Overall long-term survival after a PTLD diagnosis was about 30% in the Collaborative Transplant Study registry analysis mentioned previously (2,3).

Reduction in Immunosuppression

Reduction in immunosuppression will result in complete and durable resolution of PTLD in some cases and is typically the first treatment maneuver attempted. The same phenomenon has been described for Kaposi's sarcoma after organ transplantation. In the case of AIDS-related Kaposi's sarcoma, regression of lesions on institution of highly active antiretroviral therapy has been described anecdotally, and the incidence of this AIDS complication has clearly declined. Restoration of immune function has not had as clear an effect on the AIDS-related lymphomas. PTLD may offer some clues to possible reasons. The likelihood of response to reduced immunosuppression in an individual patient is difficult to predict. Reports have consisted of small retrospective series of patients who were not treated uniformly. In a study from the University of Pittsburgh, more than 80% of patients presenting at less than 1 year after transplantation responded to a reduction in immunosuppression, whereas none presenting at more than 1 year did so (42). More variable results with reduced immunosuppression have been reported in other series: lower response rates and greater variability in terms of the interval since transplantation (40,43). EBV is assumed to be the target of this immune response. No clear difference in the pattern of EBV antigenic expression has so far been identified in PTLD presenting early as opposed to late. Immunodominant antigens are expressed in all of the tumors, although some degree of restriction has been seen, possibly reflecting varying degrees of immune control (26,44). Tumor characteristics, specifically the presence of structural alterations conferring growth autonomy or resistance to immune-mediated destruction, may, therefore, account for the failure of immune reconstitution to eradicate the disease. In keeping with that hypothesis, the presence of mutations in the bcl-6 proto-oncogene, by use of single-strand conformation polymorphism and sequence analysis, has been shown to associate with histopathologic category and with response to reduction in immunosuppression (45). No bcl-6 mutations were identified in cases classified as plasmacytic hyperplasia. Mutations were found in 43% of polymorphic lesions and in 90% of PTLDs classified as immunoblastic lymphoma or multiple myeloma. Bcl-6 mutations were predictive for lack of response to reduced immunosuppression. Although the association was statistically significant, the correlations were retrospective and treatment was variable. Nonetheless, this finding represents the clearest predictor for response to reduced immunosuppressives to date. It would, therefore, appear that only certain PTLDs are reversible, even when immunosuppressives are reduced to the point of rejection (40).

Local Therapies

Surgical resection of PTLD can be curative. Limited-field irradiation for anatomically limited but unresectable disease has also resulted in long-term remission. Resection has furthermore been effective when a limited number of residual lesions persisted after a partial response to reduced immunosuppression (40,42). Such observations underscore the fact that PTLD differs significantly from the non-Hodgkin's lymphomas seen in the general population.

Antivirals

Regression of lymphoproliferations has been described following the use of high-dose acyclovir in a small number of cases (41,46). High-dose acyclovir has proved to be ineffective as prophylaxis for PTLT in individual bone marrow transplant recipients (47). It is not known whether other drugs, such as ganciclovir or foscarnet, are of any greater efficacy. It is also unclear whether inhibition of EBV replication could be expected to affect an established EBV-associated tumor. Uncontrolled studies (48) have suggested possible prophylactic benefit, but firm conclusions cannot be drawn in the absence of a randomized, prospective study with a no-prophylaxis control arm.

Interferon

Durable remissions have been achieved with interferon alfa 2b, given with concomitant immunosuppressives. Neither the response rate nor the mechanism of action is defined at this point. The drug might exert an antiviral and/or an antitumor effect; both early polyclonal proliferations and late-presenting monoclonal lesions have been reported to respond (47,49). In a series of 18 patients treated with interferon alfa-2b and simultaneous reduction in immunosuppression, an overall response rate of 83% (77% complete response and 6% partial response) was reported. It is unclear to what extent the responses were due to reduced immunosuppression or to interferon. Rejection and life-threatening infection occurred in half these patients, and median survival was 6 months (50).

Monoclonal Anti-B-Cell Antibodies

Anti-B-cell monoclonal antibodies have demonstrated efficacy in the treatment of PTLT. A mixture of anti-CD21 and anti-CD24 monoclonal antibodies was used in a European multicenter trial, involving both organ and bone marrow transplant recipients with PTLT (43,51). An update on this series of patients was reported (52). Fifty-eight patients with PTLT (27 following bone marrow and 31 following organ transplantation) were treated. The overall complete response rate was 61%. The relapse rate was low at 8%. The long-term overall survival was 46% (bone marrow transplant 35%, organ transplant 55%) at a median follow-up of 61 months. Complete remission was achieved in 46% of monoclonal and in 80% of oligoclonal cases ($P = .05$). Multivisceral disease, CNS involvement, and late onset PTLT (>1 year after transplant) were identified as predictive for poorer response on multivariate analysis. Only 29% of patients with CNS involvement and 22% of patients presenting later than 1 year after transplant achieved complete remission. The implications of these observations for AIDS-related lymphomas are unclear. Toxicity was mild, consisting of transient fever, hypotension, and neutropenia. The antibodies used are not currently clinically available. The commercially available anti-CD20 antibody rituximab has shown efficacy in PTLT, based on anecdotal reports (53), and on a retrospective study of 32 patients with PTLT related to organ transplant (26 patients) or to bone marrow transplant (six patients) (54). Immunosuppressives were modified in 27 patients; it is not clear whether that occurred simultaneously with antibody treatment. Among the 26 evaluable patients, 54% complete remission and 15% partial remission were reported. Median duration of follow-up was 5 months. Two relapses were seen at approximately 9 months, which is about the time when the effect of this antibody is known to

wane. In summary, monoclonal anti-B-cell antibodies appear to have significant activity in PTLT. Whether antibodies have useful activity against disease refractory to reduced immunosuppression and particularly in tumors containing structural genetic alterations remains to be clearly defined. The latter cases would be of greatest interest in attempting to extrapolate to the setting of established AIDS-related lymphomas.

Chemotherapy

Cytotoxic chemotherapy has resulted in significantly greater toxic effects in organ transplant recipients than in the general population, mainly due to infectious complications. This is analogous to what has been seen with AIDS-related lymphomas. Cytotoxics have been considered as a treatment of last resort in PTLT, as other treatment options exist. A mortality of 70% has been reported for patients presenting at more than 1 year after transplant (41,42). Septic and other complications of chemotherapy have been the major problem in some centers, whereas others have found refractory disease to be common (1,41,43). Those poor results have been obtained with a variety of full-dose or attenuated regimens, frequently combination chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone. More encouraging results have been achieved in a small series of cardiac recipients treated with aggressive chemotherapy, predominantly ProMACE-CytaBOM (40). Mortality during chemotherapy was 25% (sepsis, refractory disease), growth factor support was not used; the surviving patients all achieved complete remission. No patient has relapsed, at a median follow-up of 64 months. This regimen allowed the discontinuation of all other immunosuppressives for the duration of chemotherapy and minimized exposure to doxorubicin in cardiac recipients. This approach is currently being tested in an intergroup study being conducted by the Southwest Oncology Group and the Eastern Cooperative Oncology Group. The advent of better supportive care measures, granulocyte colony-stimulating factor, and preventive antibiotics may further reduce the toxicity of chemotherapy in this patient population. The concept that immunodeficiency-related lymphomas require aggressive rather than dose-attenuated chemotherapy is currently being studied in a number of clinical trials, including a phase II study of ProMACE-CytaBOM for AIDS-related lymphomas by the Southwest Oncology Group, and an EPOCH regimen at the National Cancer Institute (55).

T-Cell Therapy

EBV-specific immunocompetence has been rapidly restored in T-cell-depleted allogeneic bone marrow recipients by the infusion of a limited number of peripheral blood leukocytes from the donor (56). More recently, highly selective adoptive transfer of T-cell immunity has been achieved with the use of *in vitro*-expanded EBV-specific cytotoxic T cells as treatment and prophylaxis for PTLT in T-cell-depleted bone marrow transplant recipients (57,58). Polyclonal T-cell lines containing both CD4 and CD8 cells were generated, since it is not presently clear which antigens expressed by EBV-infected cells are important in generating an effector response. Adoptive transfer of EBV-specific T-cell immunity would clearly also lend itself to prophylaxis against PTLT. Whether exogenously expanded T cells would be effective in tumors refractory to reduced immunosuppression is unknown. Using such approaches in the organ transplant or AIDS setting will require significant adaptations, in

view of the major histocompatibility complex-restricted nature of the T-cell response. The vast majority of organ transplant-related PTLDs is of recipient origin (59–61), not of donor origin as is the case following bone marrow transplantation. T cells must, therefore, be generated from the patient.

In summary, significant differences and similarities exist between PTLT and AIDS-related lymphoma, in terms of viral association, histopathology and molecular pathology, clinical behavior, and response to treatment. PTLT can be a valuable model system for EBV-related lymphoid neoplasia in immunodeficiency, allowing the development and testing of screening, prophylactic, and therapeutic measures that may be directly applicable to the setting of AIDS-related lymphoma.

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NOTE

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Acquired Immunodeficiency Syndrome-Related Kaposi's Sarcoma Regression After Highly Active Antiretroviral Therapy: Biologic Correlates of Clinical Outcome

Anna Maria Cattelan, Maria Luisa Calabrò, Paola Gasperini, Savina M. L. Aversa, Marisa Zanchetta, Francesco Meneghetti, Anita De Rossi, Luigi Chieco-Bianchi

Background: Kaposi's sarcoma (KS) is the most common cancer seen in subjects with acquired immunodeficiency syndrome (AIDS). KS etiology and pathogenesis are still ill defined, and no definite improvement in survival has been obtained with current chemotherapeutic regimens. This open prospective study was aimed at evaluating the clinical response of AIDS-related KS to highly active antiretroviral therapy (HAART), a combination of protease and reverse transcriptase inhibitors, as well as the relationship between clinical response, human immunodeficiency virus type 1 (HIV-1) burden, and antibody titer against human herpesvirus 8 (HHV8) proteins. **Patients and Methods:** Fourteen KS patients were studied; 12 were in the poor-risk group. At given intervals, the patients underwent clinical examination, and their CD4⁺ cell counts, plasma HIV-1 RNA levels, and antibody titers to lytic-phase ORF65 and latent-phase HHV8 proteins were determined. **Results:** When last seen, the overall clinical response rate was 86% (median follow-up, 22 months); 10 complete and two partial responses were achieved, and two patients showed disease progression. All patients with complete or partial response showed a consistent decrease in HIV-1 RNA levels, with a corresponding increase in CD4⁺ cell counts; HIV-1 RNA levels in the two progressors remained persistently high, despite a change in HAART. HHV8 ORF65 antibody titers were generally higher in patients with extensive skin or mucosal/visceral involvement versus patients with limited disease; no differences in latent-phase HHV8 antibody titers were observed in relation to tumor burden. **Conclusion:** The findings indicate that antiretroviral therapy with protease inhibitors is effective for AIDS-related KS; the clinical response was correlated with a decrease in plasma HIV-1 RNA levels and an increase in CD4⁺ lymphocytes, whereas antibody levels to the lytic-phase HHV8 protein were influenced by the extent of tumor involvement. [J Natl Cancer Inst Monogr 2000;28:44-9]

The initial suggestion by Thomas (1) that an adaptive immune system evolved in vertebrate organisms with the principal aim of preserving cell type uniformity by eliminating spontaneously arising tumor cells was further elaborated by Burnet (2) who advanced his theory of immune surveillance. Although this innovative idea triggered many studies on tumor immunology, it also met with much skepticism and eluded many expectations of a major therapeutic breakthrough. Nevertheless, circumstantial evidence indicates that primary or acquired immunodeficiencies greatly increase the risk of tumor development and, particularly, those tumors etiologically linked with a given virus infection (3).

Kaposi's sarcoma (KS) is a good example of a tumor whose incidence is remarkably higher in immunocompromised hosts (4,5). Following its original description in 1872, KS was long regarded as a rare dermatologic condition appearing in patients of Mediterranean and eastern European origin ("classic" KS) as well as in endemic foci in sub-Saharan African areas ("endemic" KS). That KS was also associated with immunodeficiencies was noticed with the advent of therapeutically induced immunosuppression in organ and marrow transplant recipients ("iatrogenic" KS). Its incidence was dramatically augmented, however, by the onset of the acquired immunodeficiency syndrome (AIDS) epidemic in the early 1980s, and the epidemic KS variant became an AIDS-defining condition, since it was the most common malignancy in people infected by the human immunodeficiency virus type 1 (HIV-1).

Unlike classic KS, but similar to the iatrogenic variant, AIDS-related KS tends to progress rapidly and shows a wide spectrum of lesions, ranging from multiple skin patches and nodules to mucosal and visceral involvement (6). At the microscope, spindle cells separated by slits containing red blood cells are the hallmark of KS lesions; mitotic activity is moderate and a diploid profile is usually seen on flow cytometry analysis. The spindle cell phenotype recalls vascular endothelial cells, likely of lymph vessel origin, that may derive from vasoformative mesenchyme. KS tissue also contains admixed lymphocytes, hemosiderin-laden macrophages, and other inflammatory cells (4).

The etiology and pathogenesis of AIDS-related KS are still ill defined. On the basis of the natural history and histopathologic findings, it was advanced that, besides determining immunodeficiency, HIV-1 might be involved through its transactivator Tat protein, which is released by infected cells and taken up by nearby cells (7), and whose angiogenetic properties are well established (8). Moreover, HIV-1 infection might produce an increase in inflammatory cytokines, such as interleukin 1, interleukin 6, oncostatin M, and interferon gamma, which together with other angiogenic growth factors would, in turn, promote the growth of hyperplastic/neoplastic KS cells (9).

A novel herpesvirus, termed "KS-associated herpesvirus" or

Affiliations of authors: A. M. Cattelan, F. Meneghetti (Department of Infectious Diseases), S. M. L. Aversa (Department of Medical Oncology), General Hospital of Padova, Italy; M. L. Calabrò, P. Gasperini, M. Zanchetta, A. De Rossi, L. Chieco-Bianchi, Department of Oncology and Surgical Sciences, Oncology Section, AIDS Reference Centre, University of Padova, Italy.

Correspondence to: Luigi Chieco-Bianchi, M.D., Department of Oncology and Surgical Sciences, Oncology Section, AIDS Reference Centre, University of Padova, Via Gattamelata 64, 35128, Padova, Italy (e-mail: chiecoibl@u1.unipd.it).

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“human herpesvirus 8” (HHV8), has also been linked to KS (10,11). HHV8 has been detected in the spindle cells, endothelial cells, and monocytes of almost all KS lesions and in about 50% of the peripheral blood mononuclear cells of KS patients (12). Moreover, although almost 100% of the KS subjects studied had antibodies against HHV8, the HHV8 seroprevalence in the general population of the United States and Europe varies from 1% to 25%, depending on the geographic area and the methodology employed (13). The finding that HIV-1 Tat protein exerts a positive effect on HHV8 replication suggests an interplay between HIV-1 and HHV8 (14).

To define a chemotherapeutic strategy for AIDS-related KS, several studies have been conducted. Treatments with cytotoxic drugs, either as a single-agent or in combination, were found to have variable response rates and to increase the frequency of opportunistic infections with no substantial improvement in survival (15,16). The addition of antiretroviral zidovudine treatment to combination chemotherapy did not increase the response rates (17). However, the course of HIV-1 infection has been greatly modified by highly active antiretroviral therapy (HAART), a combination treatment that makes use of reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs). HAART brings about a substantial and sustained decrease in peripheral blood HIV-1 RNA levels, as well as an increase in CD4⁺ T cells, and significantly delays the development of AIDS-associated opportunistic infections and death (18). Preliminary findings suggested that PIs might also determine a reduction in KS lesions (19–23), and complete remission was recently reported in patients during HAART (24).

This study, which extends a previous report (25), was aimed at evaluating the clinical impact of HAART on AIDS-related KS lesions, as well as the relationship between the clinical response, HIV-1 viral load, and antibody titer against lytic and latent HHV8 proteins.

PATIENTS AND METHODS

Patients

From October 1996 to October 1998, all HIV-1-seropositive patients with stable or progressive biopsy-proven KS and measurable disease and who were attending the Department of Infectious Diseases of the General Hospital of Padova were enrolled in an open prospective study. At study entry, the patients underwent a physical examination that included the measurement of all cutaneous and mucosal lesions as well as the compilation of a standard body diagram; patients with a clinical suspect of visceral KS underwent gastrointestinal/bronchial endoscopy and chest tomography. The patients' disease was clinically staged according to the AIDS Clinical Trial Group (ACTG) criteria based on tumor extent (T), severity of immunosuppression (I), and other systemic HIV-1-associated diseases (S) (26,27). Performance status was determined according to the criteria of the Eastern Cooperative Oncology Group.

Study Treatment

The HAART regimen consisted of a triple-drug combination, including two RTIs and one PI, according to current guidelines (28–30). During the study, a switch from one HAART regimen to another was allowed because of intolerance or failure to reduce viral load. The drugs used in the different combinations were administered daily at the following doses: RTIs—600 mg

zidovudine, 300 mg lamivudine, 2.15 mg zalcitabine, 400 mg didanosine, and 80 mg stavudine; PIs—2400 mg indinavir, 1200 mg ritonavir, 1800 mg saquinavir, and 2250 mg nelfinavir.

Outcome End Points

During the study, a complete physical examination was done every month; tumor measurements, blood cell counts, and CD4⁺ lymphocyte counts were also recorded. If endoscopic and radiographic findings at study entry were abnormal, these examinations were repeated. Clinical responses were evaluated with the use of the ACTG criteria (26): A complete response (CR) was defined as the absence of new lesions and of any detectable residual disease, including tumor-associated lymphoedema, persisting for at least 4 weeks; for visceral KS, normal endoscopic and radiographic findings were considered a CR. A partial response (PR) was defined as the absence of new lesions and a 50% or greater decrease in the number of all pre-existing lesions, or complete flattening of 50% or more of the lesions, or a 50% or greater decrease in lesion size, determined by calculating the products of two perpendicular dimensions, for at least 4 weeks. Progressive disease (PD) was defined as the development of new lesions or an increase of 25% or more in the size of pre-existing lesions. Any response not meeting the criteria for CR, PR, or PD was considered stable disease.

Quantitative HIV-1 RNA Assay

EDTA peripheral blood samples were centrifuged at 800g for 30 minutes at 20 °C over a Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient. Plasma was recovered from the upper phase and centrifuged at 1000g for 10 minutes at 20 °C to ensure a cell-free specimen; 200 µL was employed for HIV-1 RNA determination, and the remainder was aliquoted and stored at –80 °C. HIV-1 RNA was determined with the use of a quantitative reverse transcription–polymerase chain reaction assay (Amplicor Monitor; Roche Diagnostic System, Branchburg, NJ), whose lower limit of detection is 200 HIV-1 RNA copies/mL.

Analysis of HHV8 Antibodies

Plasma samples were analyzed for antibodies to a latency-associated nuclear antigen (LANA) and a capsid-related protein encoded by ORF65, as previously described (31,32). LANA antibodies were evaluated by an indirect immunofluorescence assay on paraformaldehyde-fixed BCP-1 cells; plasma samples were initially analyzed at a dilution of 1 : 100 and subsequently at serial twofold dilutions. ORF65 antibodies were tested by the enzyme-linked immunosorbent assay (ELISA) at an initial plasma dilution of 1 : 100 and then at serial twofold dilutions; the cutoff value was the average of 10 HHV8-seronegative Italian blood donors plus 5 standard deviations. Purified recombinant dehydrofolate reductase (DHFR), the fusion partner of recombinant ORF65 protein, was employed as the control antigen; plasma samples showing reactivity to this DHFR portion were considered nonspecific by ELISA. Antibody titers were calculated as the reciprocal of the highest plasma dilution giving positive results.

Statistical Analysis

Immunologic and virologic data were analyzed by the non-parametric Mann–Whitney and Wilcoxon tests. Specimens in which the HIV-1 RNA load was below the lower detection limit of the assay were assigned a value of 100 copies/mL to include

the data in the statistical analyses. Statistical analyses were performed with the use of SAS software (SAS Institute, Cary, NC). All *P* values are two-sided.

RESULTS

Patient Characteristics at Baseline

Fourteen male patients (median age 41 years; range 28–57 years) were enrolled in this study; nine had a history of previous opportunistic infection, and KS was the AIDS-defining illness in the other five (PM, CL, AO, MA, and FM; Table 1). The median interval between KS diagnosis and study entry was 8.5 months (range, 1–47 months). At study entry, none of the patients had been treated previously with PIs. Five patients (CA, AO, ZC, MA, and FM; Table 1) had never received any antiretroviral therapy; the other nine had been previously treated with RTIs, and four of these patients (PM, SL, CL, and MS) had also received systemic KS chemotherapy (10 mg/m² bleomycin and 6 mg/m² vinblastine on days 1 and 15 every 2 weeks). After six bleomycin/vinblastine cycles, patients CL and MS were treated with bleomycin (intravenous infusion of 10 mg/m² every 2 weeks) and liposomal daunorubicin (40 mg/m² every 2 weeks), respectively. Patient PM concluded his chemotherapy 3 months prior to study entry and, at the time of enrollment, had progressive disease based on the appearance of new nodular and mucosal lesions; patients MS, CL, and SL were still under treatment at study entry.

Twelve patients were in the poor-risk group, as defined by any evidence of the following: visceral disease, tumor-associated edema, and CD4⁺ cell count of fewer than 150 cells/μL (27). Of the two patients with visceral disease, one patient (CL) had large lesions in the main left bronchial wall visualized by bronchoscopy, and the other patient (SL) had multiple pulmonary interstitial infiltrates confirmed by chest tomography. One patient (AR; Table 1) fell into the T₀I₀S₀ group; this patient had stable KS following a partial remission obtained during treatment with a dual RTI therapy 3 months prior to study entry.

At baseline, the median CD4⁺ cell count was 58 cells/μL (range, 2–443 cells/μL), and the median plasma HIV-1 RNA

level was 75 500 copies/mL (range, 2500–1 870 000 copies/mL). No statistically significant differences were observed between patients with cutaneous or limited mucosal involvement (T₀) and patients with more extensive disease (T₁) regarding both CD4 cell number (T₀ median = 75 cells/μL and range = 11–443 cells/μL versus T₁ median = 21 cells/μL and range = 2–214 cells/μL; *P* = .38, Mann-Whitney test) and HIV-1 RNA copies/mL (T₀ median = 144 000 copies/mL and range = 2500–1 870 000 copies/mL versus T₁ median = 72 000 copies/mL and range = 23 000–315 000; *P* = .90, Mann-Whitney test).

Clinical and Biologic Responses

Three patients (MS, CL, and SL) concluded their previous chemotherapies at 9, 5, and 10 months, respectively, following study entry. The initial HAART regimen was changed during the study in four patients (PM, CL, MS, and AR) because of the lack of a satisfactory virologic response and in one patient (CG) because of intolerance.

The median follow-up at last examination was 22 months (range, 8–31 months). When last seen, the overall clinical response rate was 86% (12 of 14), with 10 CRs and two PRs. The median time to CR was 6 months (range, 2–23 months); four patients with limited cutaneous lesions (MA, CW, FM, and AR) achieved a CR in a median time of 3 months (range, 2–5 months), whereas six patients with more extensive disease obtained a CR following a PR in a median time of 13 months (range, 5–23 months). All patients who achieved a CR were still in this clinical condition when last seen (Table 2).

Two patients (AO and SLu) who achieved a PR at 2 months and at 1 month, respectively, were still in this clinical condition at last examination (Table 2). Patient PM obtained a PR 6 months after HAART initiation but showed PD at 15 months. Patient MS showed PD at 9 months, despite concomitant KS chemotherapy; he was then started on paclitaxel (30 mg/m² every week) and after 2 months achieved a PR, which was followed 5 months later by PD (Table 2).

At PR, CD4⁺ cell counts were higher than baseline values (median = 36 cells/μL and range = 2–214 cells/μL versus median = 105 cells/μL and range = 27–350 cells/μL; *P* = .11).

Table 1. Characteristics of patients at baseline

Patient code	Age, y	PS*	KS staging†	Months‡	Kaposi's sarcoma (KS) lesions				CD4 cells/μL	Human immunodeficiency virus type 1 RNA copies/mL
					Visceral	Lymphoedema	Mucosal	Patch§		
PM	30	1	T ₁ I ₁ S ₁	19			+	++	2	79 000
MS	45	2	T ₁ I ₁ S ₁	47		+	+	++	4	148 000
CL	43	1	T ₁ I ₁ S ₁	9	+		+	+++	98	67 000
SL	33	1	T ₁ I ₁ S ₁	16	+		+	++	21	72 000
CR	47	2	T ₁ I ₁ S ₁	8		+		+	4	315 000
CA	49	1	T ₁ I ₀ S ₁	1		+		++	214	23 000
AO	40	2	T ₁ I ₀ S ₁	12		+		++	212	63 000
SLu	28	1	T ₀ I ₁ S ₁	8			+		40	37 000
ZC	33	1	T ₀ I ₁ S ₁	3			+		32	144 000
CG	43	1	T ₀ I ₁ S ₁	10				++	98	162 000
MA	38	1	T ₀ I ₁ S ₁	6				+	75	1 870 000
CW	57	1	T ₀ I ₁ S ₁	1				+	11	220 000
FM	36	1	T ₀ I ₀ S ₁	5				+	443	2500
AR	42	0	T ₀ I ₀ S ₀	36				+	206	29 000

*PS = performance status, determined according to Eastern Cooperative Oncology Group criteria.

†Clinical staging according to the AIDS Clinical Trial Group criteria based on tumor extent (T), severity of immunosuppression (I), and other systemic HIV-1-associated diseases (S).

‡Time from the date of KS diagnosis.

§+ = fewer than 10 patches; ++ = 10–30 patches; +++ = more than 30 patches.

Table 2. Clinical and biologic response to therapy*

Patient code	Clinical and biologic response									At last examination		
	Partial response (PR)			Complete response (CR)			Progressive disease (PD)					
	Months	CD4 cells/ μ L	HIV-1 RNA copies/mL	Months	CD4 cells/ μ L	HIV-1 RNA copies/mL	Months	CD4 cells/ μ L	HIV-1 RNA copies/mL	Response (months)	CD4 cells/ μ L	HIV-1 RNA copies/mL
PM	6	106	228 000				15	23	634 000	PD (31)	6	660 000
MS	11	27	124 000				16	51	227 000	PD (20)	81	124 000
CL	5	292	31 000	13	423	<200				CR (31)	400	<200
SL	6	101	<200	23	448	<200				CR (31)	600	<200
CR	8	119	376 000	14	234	<200				CR (17)	290	<200
CA	3	142	<200	16	247	<200				CR (24)	300	<200
AO	2	350	1000							PR (8)	520	1400
SLu	1	34	5300							PR (20)	110	7800
ZC	1	87	<200	5	148	<200				CR (27)	620	<200
CG	2	104	2300	7	196	<200				CR (21)	230	<200
MA				3	287	240				CR (10)	350	14 500
CW				2	51	<200				CR (25)	220	<200
FM				5	430	<200				CR (8)	460	<200
AR				3	276	6500				CR (30)	290	29 500

*HIV-1 = human immunodeficiency virus type 1.

Wilcoxon test), and HIV-1 RNA levels had decreased in most of the patients (median = 75 000 copies/mL and range = 23 000–315 000 versus median = 3800 copies/mL and range = 100–376 000 copies/mL; $P = .15$, Wilcoxon test), but the differences were not statistically significant. Furthermore, no statistically significant differences in the number of CD4⁺ cells and HIV-1 RNA levels ($P = .33$ and $P = .59$, respectively, Mann-Whitney test) were observed between patients who subsequently achieved a CR and those with a PR or PD.

At the time of CR, CD4⁺ cell counts were statistically significantly higher than baseline values (median = 262 cells/ μ L and range = 51–448 cells/ μ L versus median = 87 cells/ μ L and range = 4–443 cells/ μ L; $P = .006$, Wilcoxon test), and the HIV-1 RNA load had dropped to undetectable levels in eight of 10 subjects (median = 100 copies/mL and range = 100–6500 copies/mL versus baseline median = 108 000 copies/mL and range = 2500–1 870 000 copies/mL; $P = .004$, Wilcoxon test).

At last examination, an additional upsurge in the number of CD4⁺ cells was observed in almost all subjects who achieved a CR or a PR; plasma HIV-1 RNA levels were undetectable in eight subjects with a CR and relatively low in two others with a CR as well as in the two patients with a PR. However, the two patients with PD had persistently high plasma HIV-1 RNA levels and CD4 cell counts below 100/ μ L despite a change in HAART regimen (Table 2).

Antibodies against HHV8 lytic-phase ORF65 protein and LANA were determined before HAART and at different time points during follow-up. At baseline, the patients with advanced KS showed high titers of ORF65 antibodies; during HAART, small variations with no apparent relationship to the course of disease were observed (Fig. 1, panel A). All patients with T₀ disease except two (CG and AR) showed lower ORF65 antibody titers at baseline and throughout follow-up (Fig. 1, panel B). Of interest, patient CG had extensive cutaneous involvement at study entry (Table 1); he obtained a CR, and, when last seen, his titer was considerably reduced.

LANA antibodies were detected in all patients at baseline (titer range, 200–64 000), with no difference between patients with KS in stages T₀ or T₁; small variations were observed at different time points, i.e., at the PR, CR, PD, and at last examination (data not shown).

DISCUSSION

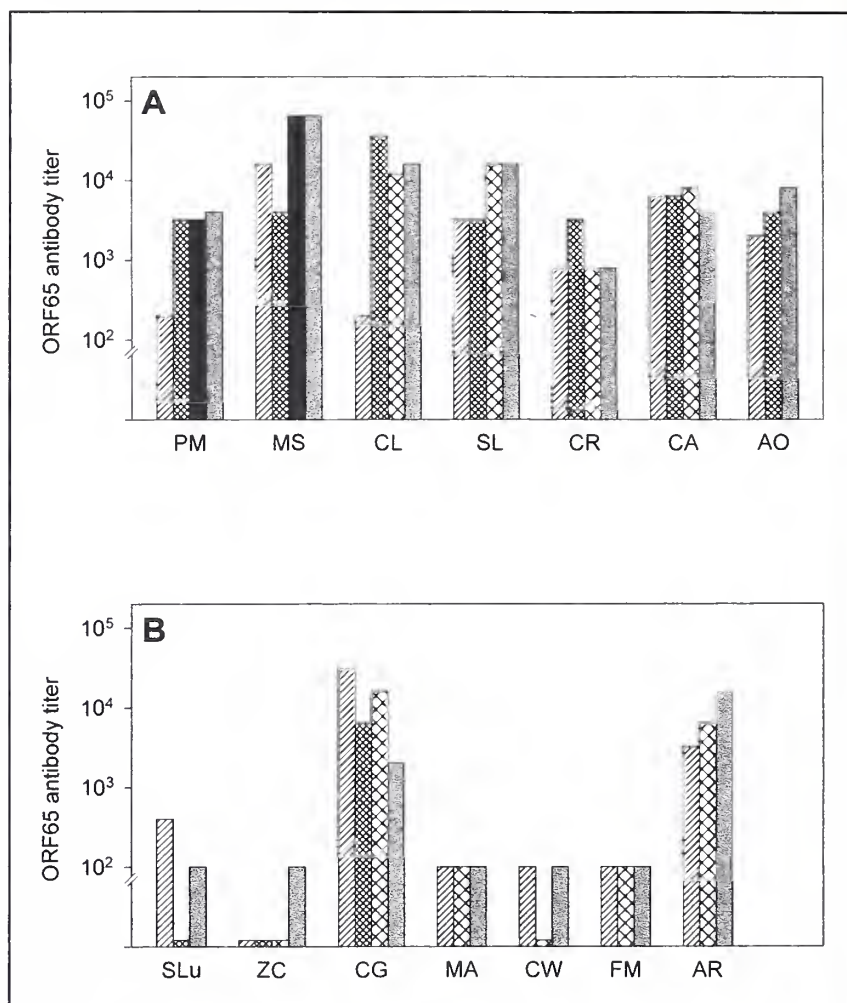
The clinical response rate to systemic chemotherapy for AIDS-related KS is usually low and short-lived (15,16); despite the use of biologic response modifiers or differentiation agents, such as interferon alfa or retinoic acid derivatives, alone or in combination with standard chemotherapy, no substantial change has emerged (33). More recent trials (34–37) have employed antiangiogenic compounds or human chorionic gonadotropin preparations in the treatment schedule; although these approaches are interesting, they need further evaluation.

In reference to HIV-1 infection, the introduction of multidrug antiretroviral regimens, based on a combination of RTIs and PIs, has greatly improved the clinical outcome, as demonstrated by a substantial decline in AIDS incidence and mortality (18). These potent antiretroviral agents induce a clearance of HIV-1 from the plasma and other biologic fluids, even if complete eradication is precluded by the persistence of latently infected cells. Moreover, immune responses to a variety of infectious pathogens are also restored in individuals who show an optimal virologic response to HAART, as evidenced by *in vitro* study findings (38) and the remarkable decrease in opportunistic infections (18).

HAART also appears to influence the clinical course of AIDS-related KS; partial or complete regressions were reported in KS patients (19–24), and our present findings are in line with these observations. In our group of 14 KS patients treated with HAART, 10 CRs and two PRs were achieved and maintained up to the last examination. We observed a substantial decrease in the plasma HIV-1 RNA load associated with a rise in the CD4⁺ cell count in all 12 patients with clinical remission as well as consistently high levels of viremia with low CD4⁺ cell counts in the two patients with progressive KS. Thus, a good correlation between efficacy of HAART and KS clinical response was evident. It is worth mentioning that eight of the 10 patients with a CR were never administered antitumor chemotherapy. It is also noteworthy that the addition of paclitaxel to the treatment schedule of patient MS caused a temporary shift from PD to a PR, even if the contemporaneous change in his HAART combination was not followed by a decrease in the plasma HIV-1 load.

Our findings indicate that the evolution of AIDS-related KS greatly depends on the entity of the HIV-1 burden and the en-

Fig. 1. Antibody titers to ORF65 protein at baseline (▨), partial response (▩), complete response (⊗), progressive disease (■), and at last examination (▨ [gray]). Antibody titer was calculated as the reciprocal of the highest plasma dilution giving positive results. **Panel A:** patients with Kaposi's sarcoma (KS) in stage T₁. **Panel B:** patients with KS in stage T₀.



suing degree of immunodeficiency. In addition, previous studies on the angiogenic properties of HIV-1 Tat regulatory protein (7) and the activation of the inflammatory cytokine cascade are consistent with the present findings, and they further emphasize the relevant, albeit indirect, role of HIV-1 infection in KS pathogenesis (9). However, the possibility that some antiretroviral PIs are also endowed with an intrinsic anti-KS activity cannot be ruled out.

Following the identification of HHV8, an increasing body of evidence has pointed to an etiologic link between this virus and KS development. Like other gamma herpesviruses, i.e., herpesvirus saimiri and Epstein-Barr virus, HHV8 also seems to possess an oncogenic potential: analysis of its genomic sequences revealed a set of genes that are structurally and functionally related to cellular genes known to interfere with cell cycle control or are endowed with growth-promoting and antiapoptotic activity (39). Furthermore, two different viral genes, K1 and K12, produced morphologic changes and focus formation indicative of neoplastic transformation when expressed in rodent fibroblasts, and they were tumorigenic *in vivo* (40,41).

We used available first-generation serologic assays to measure plasma antibody titers to lytic (ORF65) and latency-associated nuclear (LANA) antigens in an attempt to discern an antibody trend that might be indicative of HHV8 behavior during KS evolution. The LANA antibody titer showed a variable pattern; ORF65 antibody levels in general seemed to be correlated to baseline tumor extension. However, on the basis of the

present data, we cannot draw any conclusions as to whether the ORF65 antibody titers reflect the clinical evolution of KS. We expect that direct evaluation of HHV8 viremia by molecular methods would be more informative in this regard; this approach might also be useful to determine whether HHV8 expression is directly influenced by antiretroviral agents.

In conclusion, our data confirm that the HAART regimen can induce a clinical response in AIDS-related KS and provide evidence that the remission thus obtained is more prolonged than that achieved by conventional antitumor chemotherapy alone.

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NOTES

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Papillomavirus-Like Particle Vaccines

John T. Schiller, Douglas R. Lowy

Papillomavirus-like particle (VLP)-based subunit vaccines have undergone rapid development over the past 8 years. Three types are being investigated. The most basic type is composed of only the L1 major capsid protein and is designed to prevent genital human papillomavirus (HPV) infection by inducing virus-neutralizing antibodies. On the basis of positive results in animal models, clinical trials of this type of vaccine for HPV16, and other types, are currently under way. Preliminary results have been encouraging in that systemic immunization with the L1 VLPs induced high serum titers of neutralizing antibodies without substantial adverse effects. The second type of vaccine incorporates other papillomavirus polypeptides into the VLPs as L1 or L2 fusion proteins. These chimeric VLPs are designed to increase the therapeutic potential of an HPV vaccine by inducing cell-mediated responses to nonstructural viral proteins, such as E7. Studies in mice indicate that these vaccines generate potent antitumor cytotoxic lymphocyte (CTL) responses while retaining the ability to induce high-titer neutralizing antibodies. It is likely that prophylactic and therapeutic clinical trials of chimeric VLPs will be initiated in the near future. The third type of VLP-based vaccine is designed to induce autoantibodies against central self-antigens by incorporating self-peptides into the outer surface of VLPs, a process that could have therapeutic potential in various disease settings unrelated to HPV infection. In a recent proof of concept study, a peptide from an external loop of mouse CCR5 protein was inserted into a neutralizing epitope of L1. In mice, the particles generated by this chimeric L1 were able to induce high titers of CCR5 antibodies that specifically recognized the surface of CCR5-transfected cells and blocked *in vitro* infection of an M-tropic human immunodeficiency virus strain. [J Natl Cancer Inst Monogr 2000;28:50-4]

Very strong biologic, clinical, and epidemiologic evidence exists that sexually transmitted human papillomavirus (HPV) infections cause most cervical cancers (1). This infectious etiology provides an opportunity to prevent a major cause of cancer deaths in women through vaccination. The desire to prevent or treat genital HPV infection through immunization has led investigators to employ a number of strategies to develop candidate HPV vaccines (2). This report will focus on the development of one of these strategies, papillomavirus-like particle (VLP)-based subunit vaccines. In general, VLP-based vaccines are attractive for combating viral infections because they retain the highly immunogenic array of repetitive epitopes found on the surface of authentic virions, yet VLPs are devoid of the potentially harmful viral genomes. Preclinical *in vitro* and animal studies of papillomavirus VLPs, composed of only the L1 major virion protein, have moved this candidate to the forefront of vaccines to prevent HPV infection [reviewed in (3)]. They have also prompted attempts to develop second-generation VLP-based vaccines that incorporate polypeptides of other viral and cellular proteins into

the VLPs. In these cases, the VLPs are used as vehicles to facilitate immune presentation of additional antigens to both the cellular and humoral arms of the immune system (Fig. 1). Some of these second-generation vaccines are being developed with the goal of improving the effectiveness against HPV infection, whereas others have the goal of combating other diseases.

PROPHYLACTIC VACCINES

Prophylactic vaccines against viruses are thought to function primarily through the induction of virion-neutralizing antibodies that prevent infection (4). It has been difficult to employ this strategy to develop an HPV vaccine. HPV virions cannot be propagated efficiently enough in cultured cells to serve as a source of antigen for a vaccine (5). Even if they could be easily propagated, they would be unattractive as a prophylactic vaccine, because their genomes contain oncogenes. Subunit vaccines that lack the viral genome are, therefore, much more attractive candidates. However, early attempts to develop virion protein-based subunit vaccines in animal papillomavirus models were only minimally successful. This minimal success is because neutralizing antibodies predominantly recognize conformational epitopes of the L1 major capsid protein, and the early vaccines used denatured virion proteins or peptides [(6) and references therein]. The methodologic breakthrough in prophylactic vaccine development was the finding that L1 alone could self-assemble into VLPs that are structurally and antigenically very similar to authentic virions. This finding was first shown in a bovine papillomavirus type 1 (BPV1) model (7) and later confirmed for HPV VLPs as suitable serologic assays became available. VLPs have been generated in a variety of cultured cells, including those from mammals, insects, yeast, and even bacteria (3).

Because HPVs do not infect animals, studies of protection from virus challenge after VLP vaccination were conducted with the use of animal-type viruses and VLPs in their animal host species. Three animal models have been used: cutaneous challenge of domestic rabbits with cottontail rabbit papillomavirus (CRPV) (8-10), oral mucosal challenge of dogs with canine oral papillomavirus (11), and oral mucosal challenge of cattle with bovine papillomavirus type 4 (BPV4) (12). In these studies, purified VLPs were administered parenterally, and challenge virus was applied to an abraded epithelium to expose the proliferating basal keratinocytes to infection. In each model, vaccination with high nanogram to low microgram doses of L1 VLPs induced high titers of virion antibodies and protection from experimental challenge with high-dose virus. In most experiments, approximately 90% of the control subjects developed papillomas

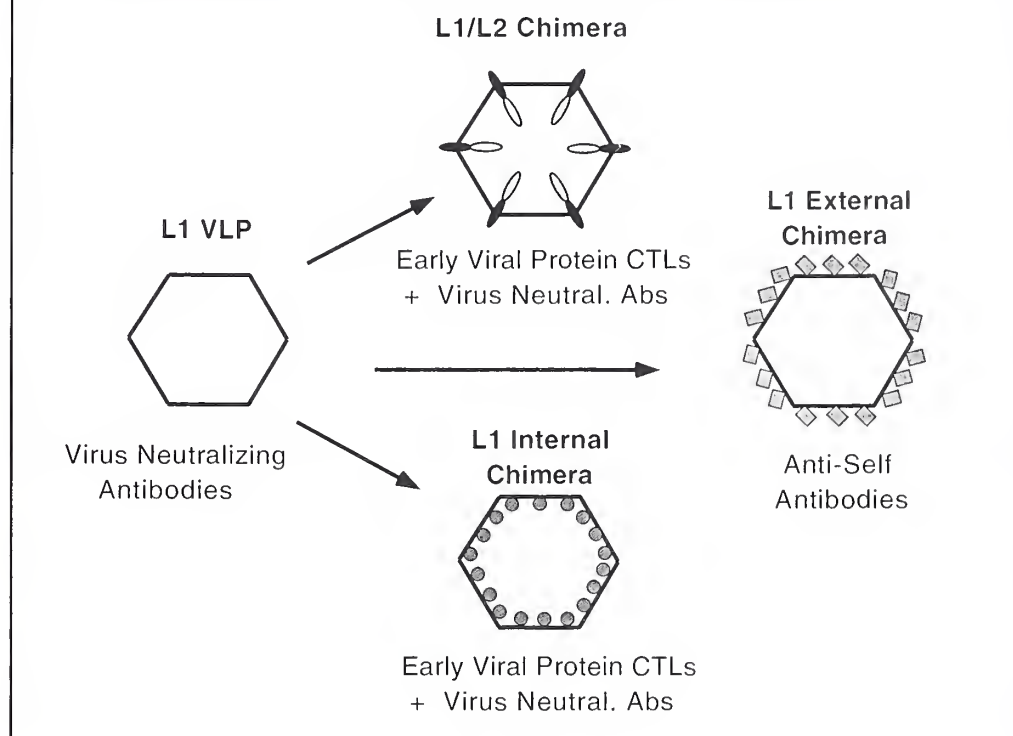
Affiliation of authors: Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD

Correspondence to: John T. Schiller, Ph.D., National Institutes of Health, Bldg. 36, Rm. 1D-32, Bethesda, MD 20892 (e-mail: schillej@dc37a.nci.nih.gov).

See "Notes" following "References."

VARIATIONS ON THE THEME OF PAPILLOMAVIRUS VLPS

Fig. 1. The type of papillomavirus-like particle is indicated above each particle. The applicable immune effector functions generated by each type of particle are indicated below. The non-virion polypeptides are depicted in gray tone.



at the site of inoculation, whereas at least 90% of the vaccinated subjects showed no evidence of infection. High-titer antibodies and protection were seen even after vaccination in the absence of adjuvant (8,9,11). However, protection was obtained only after vaccination with the homologous VLP type (8,9,11). For instance, rabbits vaccinated with BPV1 VLPs were not protected from CRPV challenge. Although the L2 minor capsid protein is incorporated into VLPs when co-expressed with L1, there were no detectable differences in the titers of virion antibodies or in the degree of protection generated after vaccination with L1 or with L1 and L2 VLPs (8,12). Protection could be passively transferred to naive animals via immune sera or purified immunoglobulin G, indicating that neutralizing antibodies were sufficient to confer protection from experimental challenge (8,11) (Table 1).

CLINICAL TRIALS

The positive results of the animal vaccine studies have prompted the National Institutes of Health (NIH), and at least two pharmaceutical companies, to begin clinical trials of HPV

VLP vaccines. The early-phase NIH trials are a collaboration of the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, and The Johns Hopkins Center for Immunization Research. They use HPV16 L1 VLPs generated in recombinant baculovirus-infected insect cells. A placebo-controlled, dose-escalation phase I trial compared intramuscular injection of the VLPs either alone, in alum, or in MF59 adjuvant (13). The vaccine was administered in three 10- μ g or 50- μ g doses at 0, 1, and 4 months. Preliminary analyses of the results (unpublished) are encouraging in that the vaccine was consistently immunogenic and well tolerated. All of the 60 subjects who received the VLPs seroconverted by 1 month after the second dose, as measured in an HPV16 VLP-based enzyme-linked immunosorbent assay (ELISA), whereas none of the 12 control subjects seroconverted during the course of the study. Preliminary analysis suggests that both adjuvants increased the titers of VLP antibodies after low-dose (10 μ g) VLP vaccination. However, at the higher dose (50 μ g), the highest geometric mean titer (GMT) was seen for the group injected with VLPs without adjuvant. The relative neutralizing titers obtained in an HPV16 pseudovirion neutralization assay appear to parallel ELISA titers for both group GMTs and individuals within groups (14). In the individuals receiving VLPs alone or VLPs plus alum, reactogenicity to the vaccine was minimal, with transient mild pain at the site of injection being the most frequent side effect. Reactogenicity did not increase with vaccine dose or boosting. The side effects in the individuals receiving VLPs plus MF59 were somewhat greater, with more frequent reports of mild or moderate transient pain at the site of injection. A phase II trial of the 50 μ g VLPs without adjuvant formulation is currently in progress.

The appropriate valency of a prophylactic HPV vaccine is

Table 1. Summary of papillomavirus-like particle (VLP) vaccine trials in animals*

Vaccine	Protection
L1 VLPs	Yes
L1/L2 VLPs	Yes
VLPs without adjuvant	Yes
Denatured VLPs	No
Heterologous VLPs	No
Immune serum	Yes

*Cottontail rabbit papillomavirus VLPs in rabbits, canine oral papillomavirus VLPs in dogs, and bovine papillomavirus type 4 VLPs in cattle.

currently under debate. On the basis of *in vitro* neutralization and hemagglutination assays of HPV VLP sera raised in animals, it is assumed that protection in people will be predominantly genotype specific (14–18). Because its goal is proof of concept, the NIH prophylactic vaccine program involves only VLPs of HPV16, the type found in approximately 50% of cervical cancers. However, many other types are also detected in cervical cancer (19), and an HPV vaccine for general distribution will likely contain multiple VLP types. Types 18, 31, and 45, along with 16, account for approximately 80% of cancers worldwide (19), so most or all of these types will likely be included in a commercial vaccine. The question of cross-interference in the elicitation of antibodies to specific VLP types in polyvalent formulations will need to be addressed during development of this type of vaccine.

Good reasons exist to consider including VLPs of nononcogenic genital HPVs in a polyvalent prophylactic HPV vaccine as well. HPV6, and HPV11 to a lesser extent, induce most genital warts (20). Although genital warts very rarely undergo malignant progression, they cause substantial morbidity. A vaccine that targets genital warts would make the vaccine more attractive to men, because men, as well as women, suffer from these lesions. In contrast, the overall incidence of HPV-induced cancers is much lower in men than in women, although a substantial proportion of penile and anal cancers in men are attributed to HPV infection (1). Vaccination of both men and women is likely to increase the effectiveness of a prophylactic vaccination program by increasing herd immunity and breaking the cycle of venereal transmission.

THERAPEUTIC VLP VACCINES

Studies in mice indicate that papillomavirus VLPs can induce L1-specific cell-mediated immune (CMI) responses (21), in addition to inducing high titers of virion antibodies. However, the virion proteins are not expressed at a detectable level in the proliferating basal keratinocytes of virus producing lesions or in the dedifferentiated cells of HPV-induced dysplasias and cancers (22). Therefore, it is unlikely that CMI responses to the virion proteins will induce regression of established lesions. In an attempt to generate effective CMI against papillomavirus-infected cells, papillomavirus VLPs have been generated in which polypeptides of nonstructural viral proteins are incorporated into the VLPs as fusion proteins of L1 or L2 [reviewed in (23)].

Chimeric VLPs that contain the entire HPV16 E7 oncoprotein fused to L2, or the N-terminus of E7 fused to L1, have been generated and shown to induce antigen-specific protection of mice from lethal challenge with E7-expressing tumor cells (24–26). Protection was obtained after a single injection of 10 µg of VLPs in the absence of adjuvant. The chimeric VLPs could also act therapeutically to induce regression of established tumors (26). The antitumor immune response to the chimeric VLPs appears to be primarily mediated by CD8⁺ cytotoxic lymphocytes. *In vitro* E7-specific cytotoxic lymphocyte (CTL) activity was detected in lymphocytes from chimeric VLP-vaccinated mice (25,26). Also, good protection was observed in major histocompatibility complex class II knockout or natural killer cell-depleted mice, but no protection was seen in β_2 microglobulin or perforin knockout mice (24). It is unclear how the VLPs are routed for class I presentation. It might involve an endocytic

pathway that the virus normally uses to enter the cell during the infectious process.

L1 and L2 chimeras for E7 produced similar results in mice, so it is unclear whether L1 or L2 chimeric VLPs would be preferable for testing in humans. L1 chimeras have the theoretical advantage in delivering more copies of the target antigen per VLP than L2 chimeras (360 for L1 versus 12 for L2). L2 chimeras have the theoretical advantage of being able to incorporate larger polypeptides and thereby increasing the number of epitopes for immune recognition. It would seem reasonable to continue testing both types of chimeras.

Several alternative strategies for generating CMI responses to E7 have been developed (2). From a safety standpoint, protein-based strategies for generating CTLs to oncoproteins, such as E7, are preferable to gene transfer-based strategies, because transfer of oncogenes might theoretically be tumorigenic. An attractive feature of VLPs is their ability to induce CTL responses without the addition of strong nonspecific immune stimulators. It is likely that early-phase trials of chimeric VLPs that contain nonstructural papillomavirus polypeptides will begin shortly. Future efficacy trials could be done in several settings. The chimeric VLPs might be effective in treating clinically apparent HPV-induced neoplastic lesions. Although the initial safety studies may be done in cancer patients, there is also considerable interest in attempting to induce regression of HPV-induced premalignant cervical dysplasias by chimeric VLP vaccination. Chimeric VLPs also have the potential to function as a combined prophylactic-therapeutic vaccine, because the insertion of the additional polypeptide did not appear to diminish the ability of the chimeric VLPs to induce high titers of virion-neutralizing antibodies (24). It is possible that chimeric VLPs could increase the effectiveness of a prophylactic vaccine by eliminating early subclinical infections that break through, despite the presence of neutralizing antibodies.

The National Cancer Institute is contemplating a prophylactic vaccine trial of an HPV16 chimera in which the entire E7 and E2 is fused to the C-terminus of L2. E2 was included because basal cells in benign lesions may express more E2 than E7, and because it simply increases the number of viral epitopes for generated CMI responses. To address concerns that a fusion protein that contains the two nonstructural viral proteins might have adverse effects on cells, mutations were introduced to inactivate the Rb binding activity of E7 and the sequence-specific transcription activating activity of E2. Fusion of E2 to E7 did not inhibit the ability of the chimeric VLPs to generate potent antitumor responses against E7 in a standard mouse tumor model (our unpublished results). A similar HPV6 chimera is being generated for eventual use in genital wart therapy trials.

Chimeric papillomavirus VLPs containing polypeptides of nonpapillomavirus targets are also being investigated in preclinical studies. One approach is to incorporate polypeptides of other sexually transmitted diseases (STDs). With the provision that induction of neutralizing antibodies is sufficient for protection against genital HPV infection, this strategy could produce a vaccine that provides protection against both HPV and another STD at little or no increase in the cost of production or administration. A second approach involves incorporating cellular tumor antigens into the VLPs. This strategy was recently shown to induce therapeutic antitumor immune responses in a mouse model (27). Immunization of mice with an immunodominant peptide derived from the P815 tumor-associated antigen P1A

induces specific T-cell tolerance, resulting in progressive outgrowth of a normally regressing P815 tumor line. In contrast, immunization with an L1 chimera that contains this same P1A peptide did not induce tolerance. Rather, it protected mice from lethal challenge with a progressor P815 line. Vaccination with this chimeric VLP also functioned therapeutically to suppress the growth of established tumors and to increase survival of the tumor-bearing mice.

AUTOANTIBODY-INDUCING VACCINES

As exemplified above, the mammalian immune system has clearly evolved to produce a strong antibody response to viruses and VLPs that mimic them. In contrast, it has evolved to normally be tolerant to self-antigens exposed to the circulating immune system. In part, the humoral immune system may distinguish between self (safe) and nonself (dangerous) on the basis of epitope arrangement, with the highly ordered repetitive arrangement of virion surface determinants being especially immunogenic (28). It was, therefore, of interest to determine whether a central self-antigen, to which the immune system was normally tolerant, could induce an antibody response if it was presented in the ordered context of a papillomavirus VLP. To test this possibility, the first external loop of the mouse CCR5 chemokine receptor (which is primarily expressed on macrophages and memory T cells) was cloned into an immunodominant-neutralizing epitope of BPV1 L1 (29). The chimeric VLPs assembled into particles, but they were smaller than those of wild-type L1 VLPs, containing an estimated 12 capsomeres rather than 72, and they did not induce BPV-neutralizing antibodies. Nevertheless, vaccination of mice expressing an identical CCR5 sequence resulted in high-titer antibodies that recognized the CCR5 peptide in ELISA. The ability of the chimeric L1 to generate CCR5 autoantibodies depended on the arrangement of the antigen, because no CCR5 antibodies were generated if the chimeric particles were denatured prior to vaccination (Table 2). The antibodies generated against the chimeric particles recognized the native CCR5, because the sera specifically bound cells transfected with mouse CCR5 and inhibited binding of RANTES, a CCR5 ligand. In contrast, antibodies generated against the same CCR5 peptide coupled to keyhole-limpet hemocyanin as a carrier bound the peptide in an ELISA but did not recognize cell surface CCR5 and did not block ligand binding, indicating that autoantibodies to the native structure were not generated by the latter immunogen.

Because human CCR5 is the co-receptor for macrophage-tropic HIV strains, it was possible to determine if the antibodies generated to CCR5 by the chimeric particles could inhibit HIV infection. Although mouse CCR5 cannot function as an HIV co-receptor, a hybrid CCR5 in which the first external loop of the mouse protein replaces the corresponding loop in the human protein can function as a co-receptor. M-tropic HIV (BaL strain) infection of cells carrying this recombinant CCR5 was effectively neutralized by sera from the chimeric VLP-vaccinated mice but not by sera from wild-type VLP-vaccinated mice (Table 2). These results establish that, in principle, mammals can be induced to synthesize neutralizing autoantibodies to virus cell-surface receptors. Whether this strategy can be effective at preventing or controlling viral infection *in vivo* remains to be determined.

The general safety of autoantibody induction as an approach to immunotherapy must obviously be considered and could vary

Table 2. Induction of CCR5 autoantibodies

Sera to	Antibody assay				
	L1 VLP ELISA	CCR5 ELISA	CCR5 FACS	HIV neutralization	BPV neutralization
L1 VLP	+	—	—	—	+
CCR5-L1 VLP	+	+	+	+	—
CCR5-L1 denatured	+	—	NT	NT	—
KLH-CCR5	—	+	—	—	NT

VLP = papillomavirus-like particle; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorter; HIV = human immunodeficiency virus; BPV = bovine papillomavirus; NT = not tested; KLH = keyhole-limpet hemocyanin.

considerably, depending on the cellular target. A potential advantage of targeting CCR5 is that it appears to be a nonessential protein. Individuals who are homozygous for a defective CCR5 gene are phenotypically normal, except that they have a substantially decreased risk of HIV infection (30,31). It is noteworthy that the mice producing CCR5 autoantibodies were outwardly healthy at 6 months after vaccination and did not exhibit signs of immunopathology at autopsy (29). There was also no decline in the numbers of macrophages or T-cell subsets that express CCR5 in comparison to control animals. Although we did not test for autoreactive T cells, we would not expect to break T-cell tolerance to CCR5. T cells that recognize central autoantigens are strongly selected against during development of the immune system. Of interest, the levels of CCR5 antibodies had begun to slowly decline by 6 months postvaccination, and the relative decline paralleled the decline in L1 antibodies for individual animals. This result suggests that exposure of the vaccinated animals to self-CCR5 does not result in continuous stimulation of the CCR5-specific B cells, presumably because the cellular protein remains in a context that continues to be ignored. The parallel decline in antibodies to the viral antigen also suggests that the presence of the cellular CCR5 does not specifically attenuate the CCR5-specific response generated against the chimeric VLPs. If autoantibody induction proves safe, this approach to immunotherapy could have diverse applications. For instance, it could potentially be an effective alternative to monoclonal antibody therapy in instances in which cell surface or soluble molecules, such as HER2/neu or tumor necrosis factor α , are known to be important mediators of disease.

In summary, papillomavirus VLP-based vaccines are being developed according to the theory that the mammalian immune system has evolved to efficiently recognize the ordered surface of nonenveloped icosahedral virions as foreign or dangerous and to generate a variety of potent immune responses to them. Therefore, vaccines that mimic the outer structural features of virions should be highly antigenic. This concept is strongly supported by the studies described in this report. Low-dose vaccination with VLPs induced both high-titer antibodies and CTLs to viral antigens without the addition of an adjuvant (Fig. 1). Even high-titer antibodies to a central self-antigen could be generated when it was presented in the context of a VLP. Given their potential for safety, it is likely that a number of clinical trials for this type of subunit vaccine will be conducted in the future.

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NOTES

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Role of the National Cancer Institute in Acquired Immunodeficiency Syndrome-Related Drug Discovery

Edward A. Sausville, Robert H. Shoemaker

The primary role of the Developmental Therapeutics Program of the National Cancer Institute (NCI) is to facilitate drug discovery by the extramural cancer and acquired immunodeficiency syndrome (AIDS) research communities. This role is accomplished in a variety of ways through grants programs, web-based informatics, provision of chemicals and natural product extracts, and screening services that will be described briefly in this article. Recently, the NCI has begun efforts to bring together molecular-targeted, high-throughput screening and extramural sites with chemical libraries of interest. This new initiative is designed to match emerging molecular targets and high-throughput assay technology with novel sources of chemical diversity in the extramural community.

Shortly after recognition of the AIDS epidemic, the Developmental Therapeutics Program (DTP) of the NCI was charged with developing a drug-screening program that might give rise to the discovery of novel therapeutics for the treatment of human immunodeficiency virus (HIV) disease. Beginning in 1987 and continuing through 1997, a functional screen for primary antiviral treatments with the use of a cell-based assay system was in place. Details of the colorimetric-assay methodology and screening strategy have been published (1,2). This screening program supported discovery of numerous lead compounds, from both natural and synthetic sources, many of which were subsequently demonstrated to be nucleoside and non-nucleoside reverse transcriptase inhibitors. A substantial number of novel natural product agents with anti-HIV activity have been isolated, including cyanovirin, a novel gp120-binding protein derived from a cultured blue-green alga (3). Detailed information on these molecules may be found on the DTP web site (<http://dtp.nci.nih.gov>), which is described below in detail.

Several novel molecules identified by the screen, or derivatives of screening leads, have been developed to the point of clinical trials. The nucleoside analog 3TC was submitted to the screen as a racemic mixture by IAF Biochem International, Inc. (Ville de Laval, Quebec), a Canadian pharmaceutical company, and subsequently licensed to Glaxo-Wellcome (Research Triangle Park, NC) for clinical development. This drug and Ziagen, a prodrug form of carbovir (4) also licensed by Glaxo-Wellcome, have been approved by the U.S. Food and Drug Administration for use in the treatment of HIV disease.

PROGRAM REVIEWS

In 1995, the National Institutes of Health (NIH) Office of AIDS Research (OAR) initiated a wide-ranging review of research activities that pertain to HIV at the National Institutes of Health. This review resulted in a number of recommendations pertinent to NCI. Prominent recommendations were that there should no longer be a focus on the mechanistically unselective cell-based screen for discovery of new AIDS-directed therapeutics and that there should be a review of the management structure and the directions that the program would be taking in the

future. The full text of this review is available at the OAR web site (<http://www.nih.gov/od/oar/>).

After receiving that report, NCI discontinued large-scale screening of synthetic compounds and natural product extracts with the cell-based assay. Information derived from this screening effort has been cataloged and made available on the DTP web site. Data for approximately 32,000 compounds are available and may be searched by biologic activity, as well as by chemical class.

The DTP AIDS program has recently undergone an additional review that was chaired by Dr. Jack Edwards of the University of California at Los Angeles and included experts from academia, industry, and the community with expertise in virology, chemistry, biology, pharmaceutical development, and clinical trials. The recommendations from this group will help shape the form of future NCI efforts in the area of HIV and AIDS-associated malignancies. This additional review follows a comprehensive review of DTP's cancer program that was initiated in 1997. The recommendations of that group, which was chaired by Dr. Susan Horwitz of Albert Einstein College of Medicine, have led to wide-ranging changes, including an emphasis on molecular targets for drug discovery and on increased interaction with the extramural drug discovery and development community. The full text of the committee's report is available on request.

CURRENT RESOURCES FOR DRUG DISCOVERY

Resources currently available from DTP to support drug discovery are summarized in Table 1. Grant support is available in the form of traditional investigator-initiated grants as well as special programs, such as the National Cooperative Drug Discovery Grants. New initiatives designed to exploit molecular targets will be discussed below. DTP maintains a web site (<http://dtp.nci.nih.gov>) that provides a wealth of information about NCI programs, chemical compounds that have been acquired and screened in anticancer and anti-HIV assays, as well as access to screening results. Tools are available on the web site to facilitate use of the information. Chemical analogue searching is possible, as is use of the COMPARE algorithm (5) for pattern recognition analysis of the anticancer drug screening database. The COMPARE program performs a correlation analysis with the use of patterns of relative *in vitro* sensitivity of 60 tumor cell lines and has been shown to be useful in helping to define the mechanism of cytotoxicity of compounds tested in the screen. The web site supports the interactive use of this program and also provides information on the chemical and natural product

Affiliation of authors: E. A. Sausville, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD; R. H. Shoemaker, Screening Technologies Branch, Developmental Therapeutics Program, National Cancer Institute-Frederick, Frederick, MD.

Correspondence to: Robert H. Shoemaker, Ph.D., Screening Technologies Branch, Bldg. 440, National Cancer Institute-Frederick, Frederick, MD 21702-1201 (e-mail: shoemaker@dtpx2.ncifcrf.gov).

See "Note" following "References."

Table 1. National Cancer Institute's resources for drug discovery

- Grants program
- Web-based informatics (<http://dtp.nci.nih.gov>)
- Chemical compounds and natural product extracts
- Cell-based screening services

extract libraries maintained by NCI. More than 100 000 crude natural product extracts derived from a wide variety of natural sources, including terrestrial plants, cultured fungi, marine organisms, and other microorganisms, are described. Two- and three-dimensional structures for more than 200 000 synthetic compounds are accessible as well as information on how to obtain samples from the repositories. A limited capacity for anti-HIV testing in the cell-based assay has been maintained and is available primarily to support research by NIH grantees.

FUTURE DIRECTIONS

During the next few years, the focus of screening and discovery activities will shift away from primarily intramurally based programs to a focus on extramurally driven activities. NCI will work toward partnering extramural principal investigators with molecular targets and sources of screening expertise with investigators developing chemical libraries. It is hoped that this partnering will occur in concert with new grant programs that DTP expects to be announced and phased in within the next year. These new grant programs will establish centers of excellence in chemical diversity as well as centers of excellence in molecular targets. They can begin to define the effects of these molecules on *in vitro* systems and then utilize NCI resources for the later stage preclinical studies that lead ultimately to clinical trial.

NCI anticipates the need to operate selected high-throughput, molecular-targeted screens at the Frederick Cancer Research and Development Center or through other contract mechanisms, either as service functions to the extramural community or in collaboration with extramural investigators with unique molecular targets. The Collaborative Research and Development Agreement (CRADA) provides a formal mechanism for defining a research plan agreeable to both NCI and a university- or industry-based investigator. The type of resources that DTP would bring to these areas would include screening expertise, a diverse repository of chemical compounds, natural products repository, expertise in bioassay-directed isolation, and characterization of leads obtained from natural products, as well as medicinal chemistry, molecular modeling, and bioinformatics resources. The resources the CRADA partner would bring to NCI would be access to state-of-the-art molecular targets and an in-depth knowledge of the biology pertinent to these systems.

As described above, the prior focus had been on HIV as a primary screening target in a cell-based assay. In the future, NCI anticipates focusing on selected molecular targets presented by HIV and extending efforts in the area of AIDS-related malignancies. These targets, associated with particular diseases, for example, angiogenesis and Kaposi's sarcoma, could also extend to aspects of immunity implicated in the expression or in the occurrence of malignancies as well as targets intrinsic to viruses associated with particular malignancies.

Presentations at this conference have alluded to potential molecular targets related to human herpesvirus 8 (HHV8) and Kaposi's sarcoma, Epstein-Barr virus and associated lymphomas, and human papillomavirus as related to the occurrence of cervical and anogenital tumors.

Because of the prevalence of Kaposi's sarcoma in AIDS patients, its clear linkage with HHV8, and the availability of com-

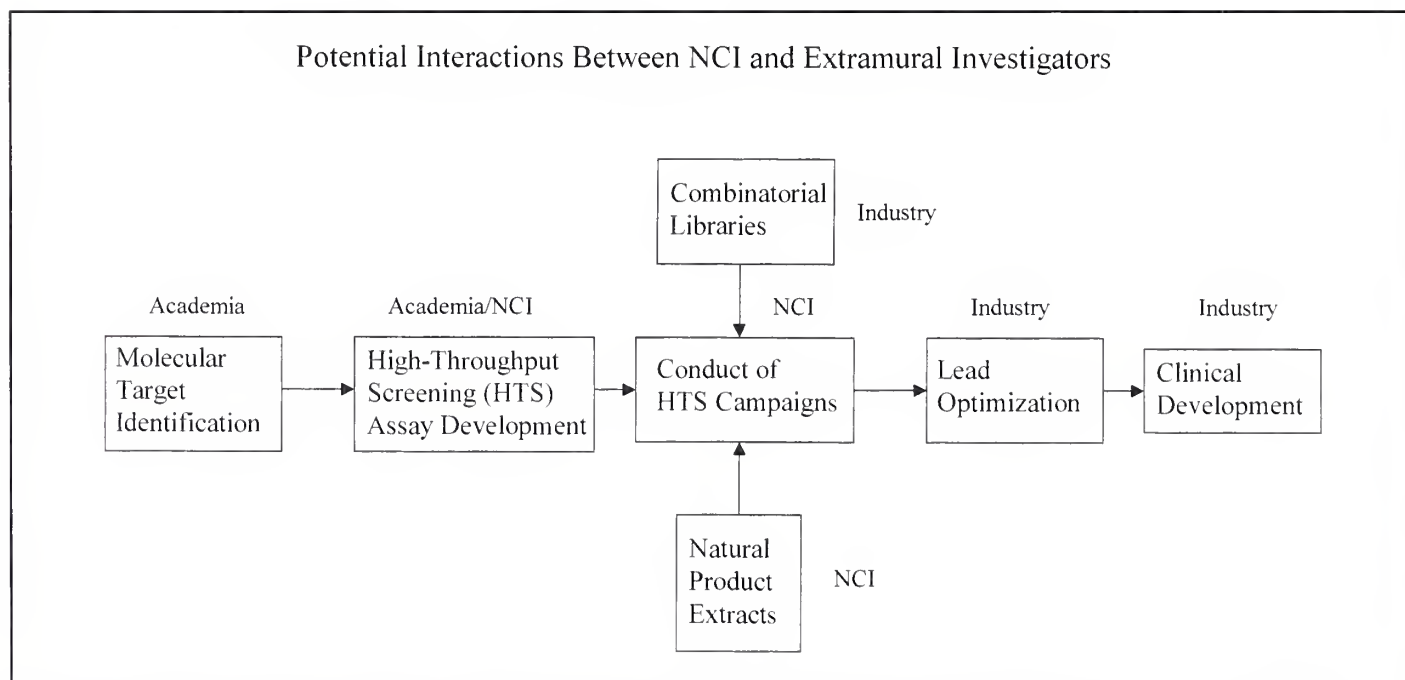


Fig. 1. The example of interactions between the National Cancer Institute (NCI) and extramural investigators depicted here is based on the University of Pennsylvania-NCI Collaborative Research and Development Agreement described in the text. Many alternative scenarios are possible. For example, academic laboratories may choose to partner with industry for conduct of high-throughput

screening campaigns. In that situation, NCI could play a role in identifying and suggesting sources of chemical diversity for input to the screen. Through the Rapid Access to Intervention Development program, an academic investigator with an advanced project might be able to obtain critical data to support clinical investigation of a new drug.

plete genomic sequence information, DTP has focused on HHV8 for the first initiatives in the area of AIDS-associated malignancies. As reported elsewhere at this conference, DTP has developed and begun to characterize a cell-based assay for testing potential anti-HHV8 agents. This assay is intended for use in conjunction with high-throughput, molecular-targeted screens.

In collaboration with Dr. Robert Ricciardi of the University of Pennsylvania (Philadelphia, PA), DTP has begun development of methods for screening for selective inhibitors of the HHV8 DNA polymerase and processivity factor. Dr. Ricciardi's group has recently cloned and characterized these genes (6), which have no counterpart in uninfected human cells and thus represent a target with potential for development of highly selective therapeutic agents. This collaboration has been structured as a CRADA in which NCI will scale-up recombinant protein expression and purification and then screen the natural product and synthetic compound repositories to identify lead structures that affect the polymerase and processivity factor. Then, in collaboration with Dr. Ricciardi's group, DTP will characterize the leads biologically in appropriate biochemical and *in vivo* models. Qualified leads may then be licensed to industry for optimization and for development to clinical trials. This collaboration illustrates one of several ways in which NCI may interact with extramural investigators who have unique molecular targets, screening technologies, or sources of chemical diversity for screening. This and other potential types of interactions are shown schematically in Fig. 1.

In addition to interaction with the extramural community, DTP anticipates continuing the association with the National Institute of Allergy and Infectious Diseases for drug discovery and development initiatives related to topical microbicides, antimicrobial leads, as well as interactions with intramural NCI laboratories engaged in research directed against relevant molecular targets, such as integrase.

NEW INITIATIVES IN DRUG DEVELOPMENT

In the area of preclinical drug development, NCI has recently launched a new program to facilitate entry of novel therapies into the clinic. The Rapid Access to Intervention Development

(RAID) program provides extramural academic investigators access to the same DTP contract resources used for development of compounds through the traditional NCI Decision Network process. Among the activities that can be supported by RAID are bulk production of drugs that would be suitable for clinical use, development of clinical formulations, or conduct of pharmacology and toxicology studies to support investigational new drug filing. For this program, NCI does not hold the investigational new drug. The intent is to remove preclinical barriers to clinical research. Additional details on the RAID program may be found on the DTP web site (<http://dtp.nci.nih.gov>).

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NOTE

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Mucosal Injury in Cancer Patients: New Strategies for Research and Treatment

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Their accomplishments were outstanding.

In addition, we thank the speakers for their expert scientific presentations and effective leadership of the often-spirited work-group discussions. Their roles in framing the science and future research directions were invaluable.

We hope the results of the conference become viewed as an important milestone relative to research for mucosal injury in cancer patients. As a result of such research, future cancer patients could likely be spared the often-serious mucosal toxic effects currently encountered in the clinical setting.

STEPHEN T. SONIS, D.M.D., D.M.Sc.
DOUGLAS E. PETERSON, D.M.D., Ph.D.
DEBORAH B. MCGUIRE, Ph.D., R.N., F.A.A.N.
DAVID A. WILLIAMS, M.D.

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nomics. Both of these content areas are potentially fertile areas for future research. As a common and representative regimen-related toxicity, mucositis is a prototypical example of how the "tail can wag the dog" with respect to QOL and cost of care. For example, while the patient's tumor may be responding well to treatment, toxicity can be sufficiently severe to preclude the patient's compliance with cancer therapy. The obvious solution to this problem rests with an adequate treatment for mucositis. In the meantime, adequate definition of QOL issues related to mucositis may provide opportunity for interim strategies. It is important that these issues become potential endpoints for clinical trials of mucositis interventions. While work to date has initially defined economic and health care costs of mucositis, there is considerable need for additional study. In particular, economic modeling for mucositis associated with a range of protocols has yet to be completed.

Scientists, health care providers, and many patients live in a dot-com society, yet there has been relatively little use of the power of the computer to archive, analyze, or model the frequency and basis for mucosal toxic effects. Use of multiagent therapies has complicated risk prediction for mucosal toxic effects. However, there is little sharing of data, short of case reports. A centralized database is an opportunity waiting to happen.

Clearly, the scope of mucosal injury provides an exciting area for innovative, imaginative research in a multiprofessional setting. We earnestly hope that this conference has provided a useful context in which to pursue these investigations.

NOTE

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Executive Summary

Douglas E. Peterson, Stephen T. Sonis

BACKGROUND AND SIGNIFICANCE

This conference was designed to generate innovative ideas that will ultimately lead to enhanced understanding of mucosal injury and strategically improved therapies for cancer patients.

There has been an impressive recent history relative to publications about and funding directed toward mucosal injury in cancer patients. In addition, professional organizations, including the International Society for Oral Oncology and the Multinational Association of Supportive Care in Cancer, have targeted mucositis as a major toxicity of cancer treatment for which new research and standards of care are needed. In this context, conference participants critically evaluated the current status of science relative to mucosal injury in cancer patients and delineated future research directions that could ultimately lead to new management strategies.

The conference format consisted of a lecture series, multiple workgroup discussions, and a summary plenary session. The research directions that emerged are summarized below. Successful pursuit of these research themes could lead to clinically important advances in the amelioration of cancer therapy-associated mucositis as well as to enhanced quality of life (QOL) for patients. The research could also potentially permit use of new, more aggressive cytoreductive cancer therapy that results in more durable remissions and improved long-term patient survival rates.

FUTURE RESEARCH DIRECTIONS

The following two principles were identified as the foundation for establishing new research directions:

- 1) The etiology, progression, and resolution of cancer therapy-associated mucositis are multifactorial in nature.
- 2) The best research model is ultimately the human model.

Specific research themes follow.

Models for the Study of Mucosal Biology, Injury, and Repair

Mucositis is an important model for the study of mucosal biology, injury, and repair. The discovery of potent agents that might protect or promote healing of the mucosal lining could lead to therapeutic, curative approaches rather than to palliation in cancer patients. Most therapeutic molecules identified to date are cytokines that affect epithelial proliferation. Basic, translational, and applied research involving the most promising molecules should be pursued.

In addition to cytokines, there is a critical need to identify and characterize other molecular interventions with similar effects. For example, current research supports the concept that significant reductions in mucositis can be achieved by appropriate manipulation of stem cell sensitivity by use of growth factors. With the identification of regulatory factors specific for the gastrointestinal tract, it is possible that the stem cells also might be more effectively regulated. New studies are required to assess

the most efficacious doses and delivery protocols, including combined and sequential use of different cellular and molecular factors.

Immune-mediated mucosal injury and repair can be investigated in substantial detail with use of *in vivo* model systems that control for antigen expression and the immune response directed toward that antigen. The following are examples:

- 1) Use of transgenic and gene-targeted mice will continue to define important mechanisms of mucosal injury, including analysis of the stages of epithelial cell damage and repair. These studies thus provide logical and relevant targets for future pharmacologic intervention. New studies are needed to further elucidate basic mechanisms of mucosal cell growth and differentiation. This research should translate these findings into patient-oriented research for treatment of inflammatory bowel disease, in addition to mucositis and other gastrointestinal complications of cancer therapies.
- 2) Mediators of cell death produced by CD8 T cells that act on intraepithelial lymphocytes can be evaluated via gene knock-out mice and blocking antibodies. Moreover, mechanisms by which tolerance versus autoimmunity is induced are readily testable in this well-defined and tissue-specific system. Future studies should focus on interaction of CD4 T cells with intraepithelial cell-expressed antigen so that new mechanisms relative to mucosal tolerance and immunity can be defined.
- 3) The gene knockout murine model also presents a unique system in which factors influencing mucosal repair can be studied. This research could directly influence the understanding of repair of epithelial damage inherent to cancer therapy. Intraepithelial cell damage can be selectively induced in enterocytes and is regulated by antigen levels and perhaps other factors such as T-cell number and viral dose. Thus, the system may be manipulated to examine the factors, immune or otherwise, involved in the repair of mucosal tissue. A further level of control can be attained by using other mucosal-specific promoters with distinct expression patterns.

Relationships between oral and gastrointestinal mucositis should be further defined, including the potential role of surrogate markers and the patient-related risk factors, including the possible role of genomics in defining risk profiles for mucositis.

Affiliations of authors: D. E. Peterson, School of Dental Medicine, Department of Oral Diagnosis, University of Connecticut Health Center, Farmington; S. T. Sonis, Brigham and Women's Hospital and Dana-Farber Cancer Institute, Divisions of Oral Medicine, Oral and Maxillofacial Surgery, and Dentistry, Boston, MA.

Correspondence to: Douglas E. Peterson, D.M.D., Ph.D., School of Dental Medicine, Department of Oral Diagnosis, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1605 (e-mail: Peterson@NSO.UCHC.EDU).

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Infection and Mucosal Injury

Intact mucosa is an important host defense against systemic infection in neutropenic patients. Conversely, mucosal injury is a significant and identifiable risk factor for localized and systemic infections, including those lesions caused by bacteria and fungi. Distinguishing between infectious-related versus regimen-related tissue damage is crucial to maintaining optimal delivery of cytoreductive cancer therapy. These principles collectively provide a basis for future research directed to several concepts, including the following:

- 1) Understanding the early steps in pathogenesis of infection at damaged mucosal sites could lead to improvements of overall outcome of cancer patients by reducing morbidity and mortality associated with both mucositis and infection.
- 2) No molecular intervention has yet been definitively proven to be effective for either prevention or treatment of mucosal injury secondary to cytotoxic cancer therapy. Furthermore, the specific mechanisms by which colonizing pathogens may amplify the severity of pre-existing mucosal damage require further study. It may be possible to bridge these scientific gaps by delineating novel, anti-infective approaches that reduce overall severity of mucosal toxicity in cancer patients by inhibiting deleterious effects of pathogenic flora at localized mucosal sites.

Mucosal Pain

Basic and clinical studies are needed to characterize the biology of pain associated with mucosal injury. Studies might include the following:

- 1) Detailed epidemiologic trials to determine patterns and severity of acute and chronic pain, as well as related side effects associated with various stomatotoxic chemotherapy and radiotherapy regimens.
- 2) Characterization of types of pain that result from oral mucosal injury as well as mucosa at other gastrointestinal tract sites.
- 3) Development of new animal models that permit evaluation of the anatomy and physiology of nociceptive processes in both normal and inflamed mucosal tissues. Emphasis should be placed on determining which inflammatory mediators activate and sensitize primary afferent nociceptors during mucosal injury.
- 4) Delineation of new clinical assessment tools for mucosal pain, including pain arising from nonoral intestinal injury.

Knowledge collectively gained from these innovative approaches can be used to develop novel therapies to decrease significant clinical problems associated with pain and its sequelae in cancer patients.

Mucosal Drug Delivery

A major challenge in formulating topical agents for the oral cavity is the need for both adhesion to moist mucosal surfaces and the maintenance of resistance to physical removal by saliva. Strategies to eliminate these research barriers should be pursued, since maximizing drug retention time at localized mucosal sites is important for improving clinical effectiveness. Use of a bioadhesive gel, for example, may reduce the frequency of application and amount of drug administered; thus, patient compliance is enhanced. In addition, lubrication and physical

protection by the bioadhesive gel often lead to reduced discomfort associated with mucositis.

Scientific findings currently exist regarding the effectiveness of transport machinery in facilitating absorption of a diverse array of therapeutic molecules into intestinal epithelial cells. In contrast, further research is needed to understand better the capacity of comparable transport processes in oral epithelial cells that are altered because of oral mucositis. Study of how best to use chemoprotective drugs to mitigate this subcellular injury is also important.

The profound effect of selected cytokines on cell proliferation requires that they be delivered locally to mucosa so as to not promote tumor growth in the patient receiving cytoreductive cancer therapy. To exert a mucoprotective effect after topical application, such compounds must transit a surface permeability barrier to reach the proliferative compartment of the epithelium. New research is needed relative to (1) preservation of high local concentrations at the mucosal surface so as to maintain a concentration gradient and (2) use of permeabilizers to ensure penetration of large molecules across the epithelial permeability barrier.

QOL and Economic Outcomes

Understanding of the effect of mucositis on QOL would be enhanced by a prospective, comprehensive, and longitudinal evaluation of mucositis severity and symptoms in relation to global and specific QOL outcomes. Such research would permit exploration of the potentially complex relationships between physician-graded mucosal injury, patient-reported specific symptom severity, and the multiple domains of QOL.

Evaluation of patient preferences for the potentially different acute and long-term consequences of increasingly aggressive cancer treatment protocols is necessary. Precise explanation of QOL implications of different therapeutic regimens may enhance treatment decision making by the patient, family, and health professionals. This may be particularly valid when there is an absence of clear survival advantage associated with the various treatment modalities under consideration.

Systematic, prospective evaluation of economic costs associated with management of mucositis is important. Cost-effectiveness and cost-benefit analyses could be conducted on the basis of the knowledge of true costs of mucositis management in relation to costs and efficacy of the preventive and therapeutic agents.

NEXT STEPS

It is essential that ongoing communication occur across relevant groups to strategically advance this research agenda. In addition to this *Journal of the National Cancer Institute* publication, specific next steps, include the following:

- 1) Posting of conference material on the Web site of the National Cancer Institute with links to the National Institute of Dental and Craniofacial Research through its National Oral Health Information Clearinghouse as well as Web sites for other relevant National Institutes of Health agencies.
- 2) Coordination of conference outcomes with the January 2002 clinical consensus conference being developed by the Mul-

tational Association of Supportive Care in Cancer and the International Society for Oral Oncology.

3) Development of a listserve of conference participants.

Continued efforts should be directed to health professional groups and patients to clarify the nomenclature for mucositis across health professional groups. This is essential for the determination of precise outcomes.

Effective integration of objective and patient-oriented outcomes of interventional clinical trials relative to federal regulatory mandates is critical. It is important to coordinate this relationship among various user groups, including academic health center investigators, clinicians, industry representatives, government officials (including those from the Food and Drug Administration and the National Institutes of Health), and patient advocate groups.

Introduction

Douglas E. Peterson, Stephen T. Sonis

A review of mucositis publications during the past 15 years reveals a number of trends. For example, there has been a quantitative leap relative to the number of publications citing mucositis in cancer models; slightly more than 100 studies appeared in the literature in 1986 in contrast to well over 400 studies in 1998. It is likely this escalating pace has been sparked by three factors: 1) recognition of mucositis as an important cancer therapy dose-limiting toxicity, 2) high incidence of mucositis in relation to optimal regimens of tumoricidal therapy, and 3) biologic complexity of the condition.

An increasing proportion of studies in recent years has evaluated mechanistic aspects of mucosal injury. Consequently, understanding of the pathophysiology of mucositis has been strategically advanced. It now seems clear that mucositis represents the endpoint of a process that includes virtually all cell and tissue types within mucosa and that is subject to alteration by local environment and genetic predisposition. Ironically and despite the number of recent studies reporting interventional clinical trials, an effective treatment for mucositis has, to date, been elusive. Thus, mucositis remains an important clinical toxicity for which novel management approaches are needed. Its diverse biologic and clinical nature lends itself to a collaborative effort to ameliorate the condition.

The proceedings reported in this monograph derive from the Conference on Mucosal Injury in Cancer Patients: New Strategies for Research and Treatment. The symposium was held in Bethesda, MD, May 24–25, 2000, and attracted an eclectic mix of clinicians and scientists that reflected a broad constituency of those interested in mucositis. Approximately one half (51%) of the 120 attendees were from either hospitals or medical or dental schools, 33% were from industry, and 16% traveled the short physical distance from the National Institutes of Health or the Food and Drug Administration. The professional training, research backgrounds, and clinical interests of participants were diverse; basic and translational scientists, radiation and medical oncologists, oral medicine specialists, nurses, general dentists, and dental hygienists all were in attendance. Geographic diversity was also a hallmark. While the majority of participants came from throughout the United States, other countries and continents including Canada, Europe, Australia, New Zealand, and Asia were also well represented.

The National Institutes of Health play a key role in determining the national agenda for biomedical research. The substantial

scientific and financial support of the conference by the National Institute of Dental and Craniofacial Research reflected recognition of the importance of mucositis as both a clinical and scientific problem.

Because of the unmet clinical needs in patients, mucosal injury has become an important niche area for pharmaceutical and biotechnological development. Thus, industry has also exhibited a leading role in driving the science that has enhanced understanding of the pathophysiology of the condition. In reality, industry will likely convert basic discovery in this area to clinically successful therapy. Industrial support, evidenced by attendance at the meeting as well as provision of financial resources, was a clear demonstration of a collective corporate commitment both to advance the field and to develop efficacious products for patients at risk for mucosal injury.

The agenda for the conference was designed to combine structured presentations in conjunction with workgroups and broad discussion. This approach, in turn, was directed to the ultimate goal of defining substantive and fruitful areas for future investigation.

Text for the formal presentations establishes the basis for this monograph. A summary of the plenary session that evolved based on workgroup proceedings also provides important perspectives for the future research directions and is included as well.

We are pleased that the conference proceedings are being published in the monograph series of the *Journal of the National Cancer Institute* (JNCI), and we thank the JNCI Editorial Board and monograph production team for their support. Access to the monograph will be available through the Web site of the National Cancer Institute (<http://www.nci.nih.gov>) and in turn from the National Oral Health Information Clearinghouse Web site (<http://www.nohic.nidcr.nih.gov>).

Affiliations of authors: D. E. Peterson, School of Dental Medicine, Department of Oral Diagnosis, University of Connecticut Health Center, Farmington; S. T. Sonis, Brigham and Women's Hospital and Dana-Farber Cancer Institute, Divisions of Oral Medicine, Oral and Maxillofacial Surgery, and Dentistry, Boston, MA.

Correspondence to: Douglas E. Peterson, D.M.D., Ph.D., School of Dental Medicine, Department of Oral Diagnosis, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1605 (e-mail: Peterson@NSO.UCHC.EDU).

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Biology of Oral Mucosa and Esophagus

Christopher A. Squier, Mary J. Kremer

The mucosal lining of the oral cavity and esophagus functions to protect the underlying tissue from mechanical damage and from the entry of microorganisms and toxic materials that may be present in the oropharynx. In different regions, the mucosa shows adaptation to differing mechanical demands: Masticatory mucosa consists of a stratified squamous keratinized epithelium tightly attached to the underlying tissues by a collagenous connective tissue, whereas lining mucosa comprises a nonkeratinized epithelium supported by a more elastic and flexible connective tissue. The epithelium is constantly replaced by cell division in the deeper layers, and turnover is faster in the lining than in the masticatory regions. Chemotherapeutic agents and radiation limit proliferation of the epithelium so that it becomes thin or ulcerated; this will first occur in the lining regions. The principal patterns of epithelial differentiation are represented by keratinization and nonkeratinization. As keratinocytes enter into differentiation, they become larger and begin to flatten and to accumulate cytokeratin filaments. In addition to the keratins, the differentiating keratinocytes synthesize and retain a number of specific proteins, including profilaggrin, involucrin, and other precursors of the thickening of the cell envelope in the most superficial layers. The concept of epithelial homeostasis implies that cell production in the deeper layers will be balanced by loss of cells from the surface. There is a rapid clearance of surface cells, which acts as a protective mechanism by limiting colonization and invasion of microorganisms adherent to the mucosal surface. [J Natl Cancer Inst Monogr 2001;29:7-15]

INTRODUCTION

The oral cavity has sometimes been described as a mirror that reflects the health of the individual. Changes indicative of disease are seen as alterations in the oral mucosa lining the mouth, which can reveal systemic conditions, such as diabetes or vitamin deficiency, or the local effects of chronic tobacco or alcohol use. Modern anticancer therapy represents a significant challenge to the integrity of the oral mucosa. Chemotherapeutic agents and radiation therapy limit the proliferative ability of the epithelium so that it becomes thin or ulcerated. This is manifest first in the more rapidly proliferating tissues, such as gastrointestinal and oral lining mucosae. There may also be indirect effects, such as damage to the salivary glands, that will reduce salivary production and impair barrier efficiency and a reduction in immunocompetence as a result of myeloablative therapy. This will increase the risk of local infection from oral organisms.

This article will first describe the organization of the oral mucosa and esophagus, then examine important functional aspects of the covering epithelium, including epithelial proliferation, differentiation, turnover, and barrier function, all of which have important implications for the maintenance of the integrity of this tissue in the face of anticancer therapy. Finally, since

most cancer is a disease of the elderly, there will be a brief consideration of changes caused by the aging of the tissue.

ORGANIZATION AND FUNCTION OF THE ORAL AND ESOPHAGEAL MUCOSA

The mucosa of the mouth and esophagus may appear to differ little from the rest of the moist lining of the gastrointestinal tract, with which it is continuous. In fact, with the notable exception of the uterine cervix, this tissue is remarkably different from other mucosae of the body and has more in common with skin, with which it forms a junction at the lips, than with the intestinal mucosa.

The soft tissues of the human oral cavity and esophagus are covered everywhere by a stratifying squamous epithelium (1). In regions subject to mechanical forces associated with mastication (i.e., the gingiva and hard palate) there is a keratinizing epithelium resembling that of the epidermis covering the skin. In these masticatory mucosae, the keratinized epithelium is tightly attached to the underlying tissues by a collagenous connective tissue, or lamina propria. The floor of the mouth, buccal regions, and esophagus, which require flexibility to accommodate chewing, speech, or swallowing of a bolus, are covered with a nonkeratinizing epithelium. The connective tissue of lining mucosae is more elastic and flexible than the connective tissue in the masticatory mucosa. The dorsum of the tongue is covered by a specialized epithelium, which can be represented as a mosaic of keratinized and nonkeratinized epithelium. This epithelium is attached tightly to the muscle of the tongue.

Fig. 1 illustrates diagrammatically the distribution of the different types of mucosa within the oral cavity (2). From measurements made by Collins and Dawes (3), it can be calculated that the masticatory mucosa represents approximately 25%, the specialized mucosa (dorsum of tongue) approximately 15%, and the lining mucosa approximately 60% of the total surface area of the oral lining.

The esophagus extends from the upper esophageal sphincter, which delineates it from the oropharynx, to the lower esophageal sphincter, representing the junction with the gastric mucosa (4). The organization of the tissues reflects their function—that of transporting ingested food from the oral cavity to the stomach. The process of peristalsis, which is initiated by swallowing and involves rhythmic contractions of the muscular walls, accomplishes this transportation. The extensibility and motility of the mucosal lining are reflected in the presence of a nonkeratinized mucosal surface resembling that of the oral lining mucosa (Fig.

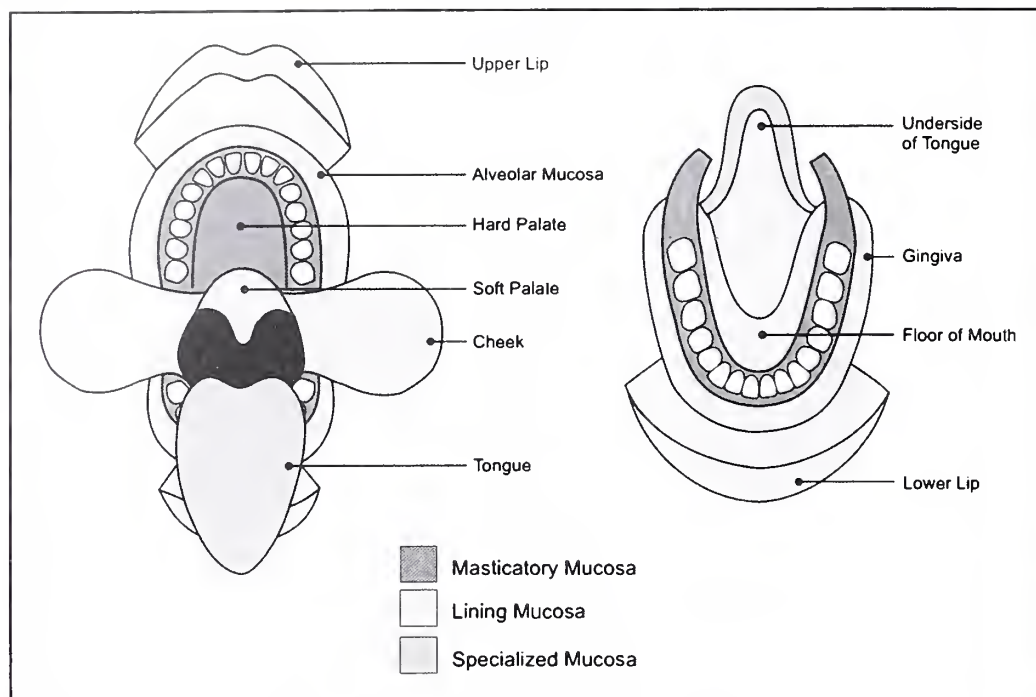
Affiliation of authors: Dows Institute for Dental Research, College of Dentistry, University of Iowa, Iowa City.

Correspondence to: Christopher A. Squier, Ph.D., D.Sc., F.R.C.Path, N419 DSB, College of Dentistry, University of Iowa, Iowa City, IA 52242 (e-mail: christopher-squier@uiowa.edu).

See "Note" following "References."

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Fig. 1. Diagram to show the anatomic location and extent of masticatory, lining, and specialized mucosa in the oral cavity. [Modified from reference (2).]



2). This surface is separated from the submucosa by a muscularis mucosa, consisting of a smooth muscle and elastic fiber layer, which may serve to reduce the excursion of the luminal lining mucosa as a result of the contractions of the external esophageal muscle, consisting of circular and transverse layers of striated or smooth muscle.

The primary function of oral and esophageal epithelium is the protection of the underlying tissue (1). In the masticatory regions, the mechanically tough stratum corneum serves to dissipate shearing forces, and in the lining areas, including the esophagus, there is a distensible and flexible surface layer. In both regions, lipid-based permeability barriers in the outer epithelial layers protect the underlying tissues against fluid loss and against the ingress of a range of potentially harmful environmental agents. These include microbial toxins and enzymes and antigens and carcinogens from foods and beverages.

STRUCTURE OF THE ORAL AND ESOPHAGEAL MUCOSA

All covering and lining tissues of the body consist of a surface epithelium supported by a fibrous connective tissue. Epithelium, by virtue of the close packing and constant turnover of cells, is well adapted to protect underlying tissues and organs against mechanical and chemical insult, whereas the connective tissue, consisting of relatively few cells in an extensive matrix, provides mechanical support and nutrients for the epithelium. In comparing the structure of skin and oral mucosa to the gastrointestinal tract, a major difference emerges in the organization of the epithelium, which reflects the different functions of these regions. The lining of the stomach and small and large intestine consists of a simple epithelium composed of only a single layer of cells, which facilitates absorption across the tissue. Skin, oral mucosa, and esophagus are covered by a stratified epithelium (Fig. 3) composed of multiple layers of cells that show various patterns of differentiation (or maturation) between the deepest cell layer and the surface.

Features that distinguish the oral and esophageal mucosa

from skin are its moist surface and the absence of appendages. The skin contains numerous hair follicles, sebaceous glands, and sweat glands, whereas the glandular component of oral and esophageal mucosa is represented primarily by the minor salivary glands. These glands are concentrated in the submucosa, and the secretions reach the mucosal surface via small ducts. The salivary glands have an important role in maintaining a moist surface containing mucins and a variety of antimicrobial substances as well as epidermal growth factor (EGF). In the esophagus, the minor salivary glands can produce a secretion with high bicarbonate concentration to neutralize refluxing stomach acid (5). Sebaceous glands are present in the upper lip and buccal mucosa in about three quarters of adults. Unlike the esophagus,

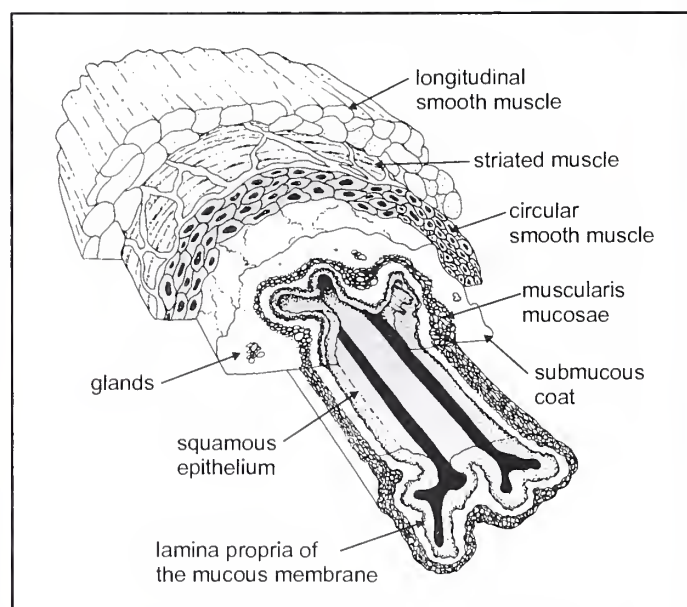


Fig. 2. The organization of the tissues of the human esophageal lining. [Modified from reference (4).]

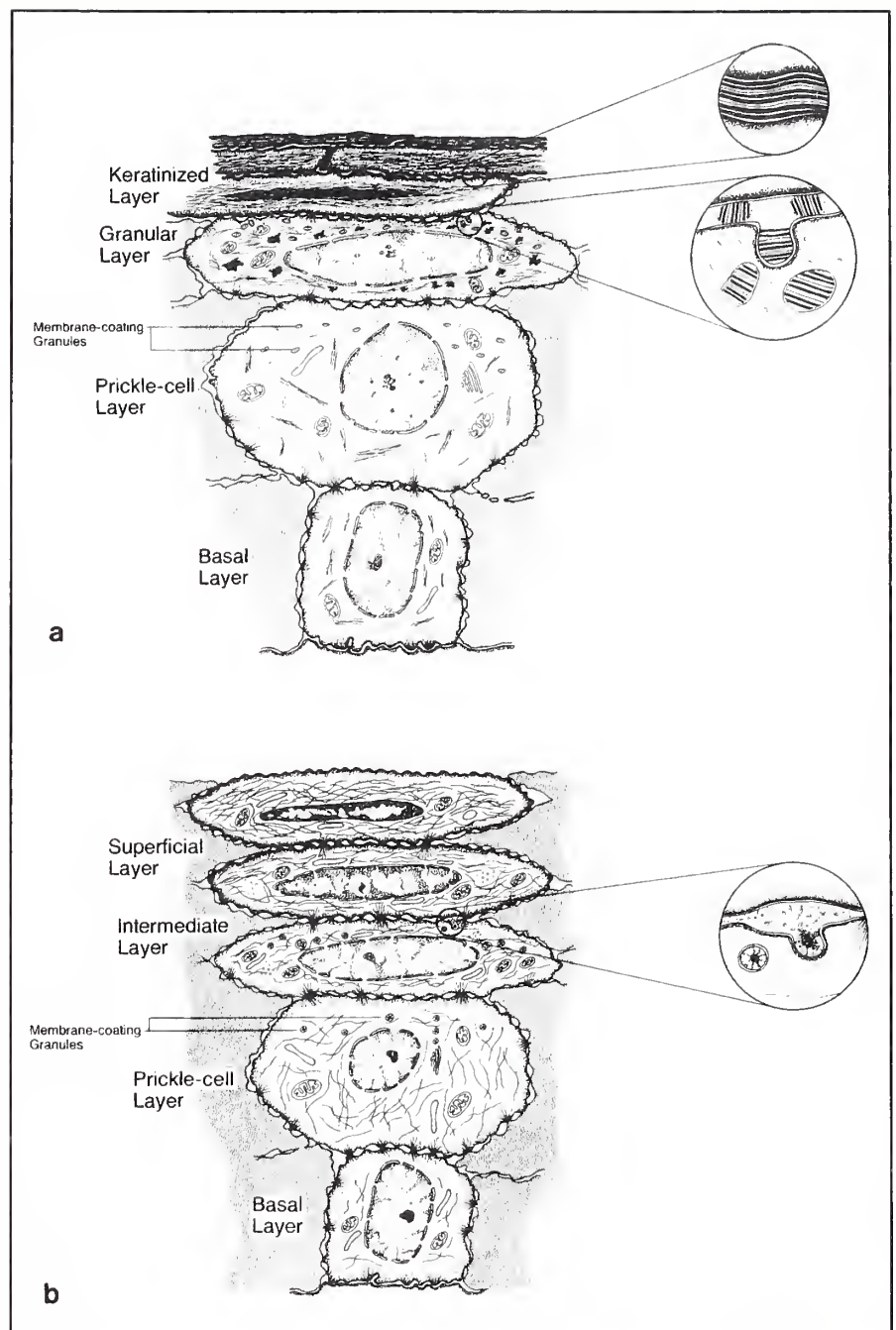


Fig. 3. Principal ultrastructural features of differentiation in (a) keratinized oral epithelium and (b) nonkeratinized oral and esophageal epithelium. [Modified from reference (48).]

the oral mucosa has no muscularis mucosae, and, consequently, it is difficult to identify clearly the boundary between it and the underlying tissues. In many regions, such as the cheeks, the lips, and parts of the hard palate, a layer of loose fatty or glandular connective tissue containing the major blood vessels and nerves supplying the mucosa separates the oral mucosa from underlying bone or muscle. This represents the submucosa in the oral cavity, and its composition determines the flexibility of the attachment of the oral mucosa to underlying structures. A similar organization is seen in the esophagus. In regions of the oral mucosa, such as the gingiva and parts of the hard palate, the oral mucosa is attached directly to the periosteum of underlying bone with no intervening submucosa. This arrangement is called a mucoperiosteum and provides a firm, inelastic attachment. In several regions of the oral cavity, there are nodules of lymphoid tissue

consisting of crypts formed by invagination of the epithelium into the lamina propria. These areas are extensively infiltrated by lymphocytes and plasma cells. Because of their ability to mount immunologic reactions, such cells play an important role in combating infections of the oral regions.

The mucosal lamina propria consists of cells, blood vessels, neural elements, and fibers embedded in an amorphous ground substance. The lamina propria shows regional variation in the proportions of its constituent elements, particularly in the concentration and organization of the fibers. Cancer therapies will tend to lower cell proliferation and turnover in connective tissue; ionizing radiation has a direct effect on large molecules that make up the ground substance, so that depolymerization occurs, vascular permeability increases, and there will be tissue edema and an inflammatory infiltrate (6). Damage to fibroblasts will

result in cell loss and the appearance of abnormal cells leading to fibrosis after about 6 months (7). Similarly, damage to blood vessels will lead to hypovascularity and tissue ischaemia (6). Together, these changes will reduce the ability of the tissue to heal and resist infection (8).

CELLULAR AND MOLECULAR EVENTS IN DIFFERENTIATION IN ORAL AND ESOPHAGEAL EPITHELIUM

The effects of cancer therapy primarily manifest in the oral and esophageal mucosae as changes in the epithelium that reflect damage to proliferating and differentiating cells. This section will describe keratinocyte structure and function in normal tissue. Chemotherapeutic agents and radiation therapy limit the proliferative ability of the epithelium so that it becomes thin or ulcerated. Basal keratinocytes are cuboidal or columnar cells with a bounding plasma membrane and a full complement of the normal intracellular organelles (Fig. 3). These cells are capable of division so as to maintain a constant epithelial population as cells are shed from the surface. Tissue homeostasis requires differentiation and desquamation at the epithelial surface to be matched by cell division. Many factors, including aging and disease, can alter this balance so that an epithelium may become thicker (hyperplastic) or thinner (atrophic) than normal.

The progenitor cells are situated in the basal layer in thin epithelia, such as the floor of the mouth, and in the lower two to three cell layers in thicker epithelia, such as the cheek, esophagus, and palate. Dividing cells tend to occur in clusters so that more are seen at the bottom of epithelial ridges than at the top. The progenitor compartment is not homogeneous but consists of two functionally distinct subpopulations of cells. A small population of progenitor cells cycles very slowly and is considered to represent stem cells whose function is to produce basal cells and retain the proliferative potential of the tissue (9–11). Because it divides infrequently, the epithelial stem cell may be important in preserving the genetic information of the tissue, since DNA is most vulnerable to damage during mitosis. While the position of stem cells can be related to anatomic structure in some tissues, such as intestine, tongue papillae, and hair follicles, the cells are not morphologically identifiable in most areas of skin and oral mucosa. There have been many attempts to develop specific stem-cell markers, including the presence of adhesion molecules, such as the $\beta 1$ -integrins, β -catenin, and cytokeratins 15 and 19 which some have claimed can be used to identify these cells in skin and oral mucosa (12–15). The larger portion of the progenitor compartment is composed of amplifying cells whose function is to increase the number of cells available for subsequent maturation by entering into mitosis.

The control of epithelial proliferation and maturation is the subject of extensive research, and there are a large number of biologically active substances, most of which are peptide growth factors that are collectively termed cytokines and that may stimulate or suppress epithelial cell proliferation. Those that stimulate keratinocyte proliferation include epidermal growth factor (EGF), transforming growth factor- α (TGF- α), platelet-derived growth factor (PDGF), and interleukin 1 (IL-1) (16–18). The rate of proliferation is the result of interaction between positive and negative regulators, which act via a complex control system involving the binding of peptide factors to cell surface receptors, a cascade of cytoplasmic elements regulated by the activities of kinases and phosphatases, and transcriptional activ-

ity in the nucleus leading to expression of proteins involved in cell cycle regulation (18,19).

Mitotic activity can also be affected by a number of factors, such as time of day, stress, and inflammation. For example, the presence of a slight subepithelial inflammatory cell infiltrate stimulates mitosis, while severe inflammation causes a marked reduction in proliferative activity. It has recently been demonstrated that, for buccal epithelium, there is a clear circadian rhythm, with most cells being in the mitotic (M) phase at 2100 hours (19). Since the M phase represents one of the most radio-sensitive stages of the cell cycle, radiation therapy involving the oral mucosa should optimally be administered in the morning.

The use of different techniques has led to a wide range of estimates of the rate of cell proliferation in the various epithelia, but, in general, the rate is highest for cells in the thin nonkeratinized regions, such as floor of mouth and underside of tongue, than for the thicker keratinized regions, such as palate and gingiva (20) (see Table 1). Apart from measuring the number of cells in division, it is also possible to estimate the time necessary to replace all of the cells in the epithelium. This is known as the turnover time of the epithelium and is derived from knowledge of the time it takes for a cell to divide and pass through the entire epithelium. Published human data for turnover times range from a median value of 34 days for epidermis to 4 days for the small intestine, with the values for oral and esophageal epithelium falling between (21,22) (see Table 1). The regional differences in the patterns of epithelial maturation appear to be associated with different turnover rates; for example, nonkeratinized buccal epithelium turns over faster than keratinized gingival epithelium. Such differences can have important implications for healing and for the rate of recovery of the tissue from damage, which is of particular relevance in considering the effects of cancer therapy on these regions. Clinically, these differences are reflected both in the more rapid appearance of therapy-induced mucositis than in dermatitis and in the prevalence of damage to nonkeratinized rather than to keratinized surfaces.

After cell division, each daughter cell either recycles in the progenitor population or enters the maturing compartment. The switch between proliferation and differentiation is modulated by the presence of factors, such as extracellular calcium, phorbol esters, retinoic acid, and vitamin D3 (23). Cells in the basal layer are attached by integrin-containing focal adhesions, and differentiation involves migration with a loss of integrin expression and an increase in cadherin-mediated adhesion via close intercellular junctions or desmosomes. There are also changes in the

Table 1. Epithelial cell proliferation and turnover in selected tissues

Tissue region	Mean labeling index, %*	Median turnover time, days†
Small intestine	—	4
Floor of mouth	12.3	20
Labial mucosa	11.8	—
Buccal mucosa	10.2	14
Ventral tongue	10.1	—
Esophagus	—	21‡
Gingiva	9.1	—
Hard palate	7.2	24
Dorsal tongue	4.3	—
Skin	—	27

*Reference (19).

†Reference (20).

‡Reference (21).

composition of intracellular proteins, termed cytokeratins, and in the development of new ones, including involucrin, loricrin, and filaggrin (24,25).

The principal patterns of differentiation are represented by keratinized and nonkeratinized epithelia. Differentiation in keratinized epithelia (Fig. 3, a) leads to production of the stratum corneum. The cornified cells making up this layer are flat and hexagonal in shape (26), filled with a compact array of condensed cytokeratin filaments (27), bounded by a thickened cell envelope (28), and surrounded by an external lipid matrix (29,30).

As cells leave the basal layer and enter into differentiation, they become larger and begin to flatten and accumulate cytoplasmic protein filaments, representing the cytokeratins. Keratins represent 30 different proteins of differing molecular weights; those with the lowest molecular weight (40 kd), such as keratins 8 and 18, are found in glandular and simple epithelia; keratins of intermediate molecular weight are found in stratified epithelia; and the largest keratins (approximately 67 kd) are found in keratinized stratified epithelium. All stratified oral epithelia possess keratins 5 and 14 in the undifferentiated basal cells, but differences emerge in the suprabasal layers with differentiation. Ortho-keratinized oral epithelium, such as the palate, contains keratins 1 and 10, whereas gingiva and parakeratinized palatal epithelium contains keratins 1 and 10 or keratins 4 and 13. Nonkeratinized epithelium, including esophagus, contains keratins 4 and 13 (31,32).

As the cells enter the prickly cell layer, small organelles known as membrane-coating granules or lamellar granules representing accumulating lipid become evident (Fig. 3, a) (33). In addition to the accumulation of lipids and keratins, the differentiating keratinocytes synthesize and retain a number of specific proteins, including profilaggrin (34,35), involucrin (36), and other precursors of the thickening of the cell envelope (37). At the boundary between the granular and cornified layers, the membrane-coating granules migrate to the superficial (apical) aspect of the keratinocyte, where the bounding membrane of the organelle fuses with the cell plasma membrane so that the lipid lamellae are extruded into the extracellular spaces of the surface layer (28,29). Thus, the membrane-coating granules are believed to be responsible for the formation of a superficial, intercellular, permeability barrier in stratified squamous epithelium. After the granules are extruded, the interior of the cell becomes filled with aggregated cytokeratin filaments, and involucrin, loricrin, and other proteins are deposited on the inner aspect of the plasma membrane as a thick band of protein that becomes covalently cross-linked (24,25).

In keratinized oral epithelium, about 50% of the intercellular space of the stratum corneum is occupied by desmosomes (38), and the interdesmosomal regions are frequently dilated. Although the extruded membrane-coating-granule contents fuse to form multiple broad lipid sheets in the intercellular spaces of the stratum corneum of this tissue, the number of individual lamellae in oral tissue is less than that observed in epidermis.

In nonkeratinizing epithelia (Fig. 3, b), the accumulation of lipids and of cytokeratins in the keratinocytes is less evident and the change in morphology is far less marked than in keratinizing epithelia. The mature cells in the outer portion of nonkeratinized epithelia become large and flat and possess a cross-linked protein envelope, but they retain nuclei and other organelles, and the cytokeratins do not aggregate to form bundles of filaments, as

seen in keratinizing epithelia. As cells reach the upper one third to one quarter of the epithelium, membrane-coating granules become evident at the superficial aspect of the cells and appear to fuse with the plasma membrane so as to extrude their contents into the intercellular space. The membrane-coating granules found in nonkeratinizing epithelia are spheric in shape and membrane bounded and measure about 0.2 μm in diameter (39). They have often been referred to as cored granules because of their appearance in transmission electron micrographs. Such granules have been observed in a variety of human nonkeratinized epithelia, including oral mucosa (40–42), esophagus (43), and uterine cervix (44). Studies employing ruthenium tetroxide as a postfixative have indicated that a small proportion of the granules in nonkeratinized epithelium do contain lamellae, which may be the source of short stacks of lamellar lipid scattered throughout the intercellular spaces in the outer portion of the epithelium (45). In contrast to the appearance of the intercellular spaces of the surface layer of keratinized epithelia, those of the superficial layer of nonkeratinizing epithelia contain electron lucent material, which may represent nonlamellar phase lipid, with only occasional short stacks of lipid lamellae. It is the absence of organized lipid lamellae in the intercellular spaces that accounts for the greater permeability of this tissue.

The concept of epithelial homeostasis implies that cell production in the deeper layers will be balanced by loss of cells from the surface. While there has been much focus on programmed cell maturation and death (e.g., apoptosis) in other systems, comparatively little is known about the events determining desquamation in skin and mucosa. The available evidence suggests a programmed breakdown of cell adhesion molecules, involving both lipids and proteins, probably by intercellular enzymes that might originate in the extruded membrane-coating granules (46). Regardless of the nature of the process, the rate at which cells leave the surface represents a defense mechanism by rapidly clearing the substrate to which many microorganisms adhere so that they are unable to produce toxic effects or to invade. Data for murine oral mucosa from Kvidera and Mackenzie (47) suggest a clearance of surface cells in 2–4 hours, depending on the region. While these rates are likely to be lower in humans, the process will clearly limit colonization and invasion.

NONKERATINOCYTES IN ORAL AND ESOPHAGEAL EPITHELIUM

Many histologic sections of oral and esophageal epithelium contain cells that differ in appearance from the other epithelial cells, and it is obvious from ultrastructural and immunochemical studies that they represent a variety of different cell types, including pigment-producing cells (melanocytes), Langerhans' cells, Merkel cells, and inflammatory cells such as lymphocytes, which together can make up as much as 10% of the cell population in the oral epithelium (48). All of these cells except Merkel cells lack desmosomal attachments to adjacent cells, so that during histologic processing, the cytoplasm shrinks around the nucleus to produce the clear halo. None of these cells contain the large numbers of tonofilaments and desmosomes seen in the epithelial keratinocytes nor do they participate in the process of maturation seen in oral epithelia; therefore, they are often collectively called nonkeratinocytes.

The endogenous pigments most commonly contributing to the color of the oral mucosa are melanin and the hemoglobin in the blood. Melanin is produced by the specialized pigment cells called melanocytes, which are situated in the basal layer of the oral epithelium and the epidermis. Melanocytes lack desmosomes and tonofilaments but possess long dendritic processes that extend between the keratinocytes, often passing through several layers of cells. Melanin pigment is synthesized within the melanocytes as small structures called melanosomes. These are inoculated or injected into the cytoplasm of adjacent keratinocytes by the dendritic process of the melanocyte. Similar cells have been described in the esophageal epithelium and can give rise to melanotic lesions (49).

Another type of dendritic cell sometimes seen in the suprabasal layers of epidermis and oral and esophageal epithelium is the Langerhans' cell (48,50). It is usually demonstrated by specific immunochemical reactions that stain cell surface antigens. Langerhans' cells may be capable of limited division within the epithelium, but it is clear both that they can move in and out of the epithelium and that the source of these cells is the bone marrow. This is in accord with evidence suggesting that they have an immunologic function, recognizing and processing antigenic material that enters the epithelium from the external environment and presenting it to helper T lymphocytes. It also seems likely that Langerhans' cells can migrate from epithelium to regional lymph nodes.

The Merkel cell is situated in the basal layer of the oral and esophageal epithelium and epidermis (48,51). It possesses keratin tonofilaments and occasional desmosomes linking it to adjacent cells, but the characteristic feature of the Merkel cell is the presence of small, membrane-bound vesicles in the cytoplasm, sometimes situated adjacent to a nerve fiber associated with the cell. These granules may liberate a transmitter substance across the synapse-like junction between the Merkel cell and the nerve fiber and, thus, trigger an impulse. This arrangement is in accord with neurophysiologic evidence suggesting that the Merkel cell is a sensory cell responding to touch. Merkel cells may arise from division of an epithelial cell (keratinocyte).

Inflammatory Cells

When sections of epithelium taken from clinically normal areas of mucosa are examined microscopically, a number of inflammatory cells can often be seen in the nucleated cell layers. These cells are transient, and the cell most frequently seen is the lymphocyte, although the presence of polymorphonuclear leukocytes and mast cells is not uncommon. Lymphocytes are often associated with Langerhans' cells, which are able to activate T lymphocytes.

It is becoming evident that the association between nonkeratinocytes and keratinocytes in skin and oral mucosa represents a subtle and finely balanced relationship in which cytokines represent the controlling factors (16). Thus, keratinocytes produce interleukins (1, 6, 7, 8, 10, 11, and 12), colony-stimulating factors (GM, G, and M), and tumor necrosis factor- α , all of which modulate the function of Langerhans' cells. In turn, Langerhans' cells produce IL-1, which can activate T lymphocytes, which secrete IL-2, thus bringing about proliferation of T cells capable of responding to antigenic challenge. IL-1 also increases the number of receptors to melanocyte-stimulating hormone in

melanocytes and so can affect pigmentation. The influence of keratinocytes extends to the adjacent connective tissue where cytokines produced in the epithelium can influence fibroblast growth and the formation of fibrils and matrix.

EPITHELIAL SURFACE BARRIER

In a variety of stratified squamous epithelia, there is an effective permeability barrier in the tissue. For example, present in the oral mucosa and esophagus is an abundant flora containing many opportunistic organisms, yet inflammatory lesions are relatively infrequent, except around the teeth. The location of this barrier in the superficial layers of the epithelium has been confirmed by experiments that demonstrate an increase in permeability when the surface layers are removed by stripping (52). Studies with microscopically visible tracers, such as small proteins (53) and dextrans (54), suggest that the major pathway across stratified epithelium of large molecules is via the intercellular spaces and that there is a barrier to penetration as a result of modifications to the intercellular substance in the superficial layers, described in the previous section. However, it is clear from measurements of permeability that different compounds may penetrate an epithelium at different rates, depending on the chemical nature of the molecule and the type of tissue being traversed. This has led to the suggestion that materials with different chemical properties cross the barrier region by different routes, some crossing the cell membrane and entering the cell (transcellular or intracellular route) and others passing between the cells (intercellular route). For oral mucosa, Squier and Lesch (55) have used light and electron microscopic autoradiography to show the route taken by isotopically labeled compounds applied to the surface of the tissue. Compounds ranging from water to cholesterol, applied to either keratinized or nonkeratinized oral epithelium, could be subsequently localized in the intercellular regions of the superficial layer of the tissues, suggesting that this compartment is the predominant route for compounds moving across the barrier layer of oral epithelium. However, Zhang and Robinson (56) have pointed out that the pH dependency that is evident in absorption of ionizable compounds reflects their partitioning into the epithelial cell membrane, so it is likely that such compounds will tend to penetrate transcellularly. Finally, from the point of view of delivering bioactive peptides that might protect the epithelium during cancer therapy, it is worth noting that the superficial layers of the tissue may act as a reservoir for topically applied compounds. Although this phenomenon has been inferred from kinetic studies in oral mucosa (57,58), it is poorly understood. As we have already mentioned, the permeability barrier in nonkeratinized epithelia consists of groups of lipid lamellae located in the intercellular spaces of the superficial epithelial layer (45,59). These limit the penetration of nonpolar compounds, which may become trapped in a nonlipid or fluid lipid intercellular compartment of the barrier layer. Thus, the surface layer of the epithelium may take up a compound relatively rapidly (depending on its lipophilicity and the nature of the vehicle). Once saturated, this layer cannot adsorb any more material, regardless of the duration of exposure. Subsequently, the adsorbed material diffuses into the deeper layers of the tissue at a fairly constant rate that is more dependent on the capacity (or loading) of the reservoir than on the duration of surface exposure.

The constancy of the oral environment is ensured to a large extent by the continual secretion of saliva into the oral cavity

from the three major salivary glands and numerous minor salivary glands located in, or beneath, the mucosa. Saliva, by continually bathing the surface of the oral mucosa, maintains a moist atmosphere and a stable, but slightly acidic, pH. Compared with the secretions of the gastrointestinal tract, saliva is a relatively mobile fluid with less mucin, limited enzymatic activity, and virtually no proteases. The mucosal surface has a salivary coating that has been estimated to be 70 nm thick (3) and that may act as an unstirred fluid layer. Several independent lines of evidence suggest that saliva and salivary mucin contribute to the barrier properties of oral mucosa (60). Within saliva there is a high-molecular-weight mucin named MG1 (61) that can bind to the surface of the oral mucosa so as to maintain hydration, provide lubrication, concentrate protective molecules such as secretory immunoglobulins, and limit the attachment of microorganisms. Histatins are small salivary-derived histidine-rich polypeptides with marked antifungal activity (62). These may be augmented by the activity of a recently discovered class of antimicrobial peptides, the defensins, that are expressed by oral epithelium (63).

AGING OF ORAL MUCOSA

Skin shows well-documented changes in structure and function with age, most of which arise from chronic exposure to UV radiation (i.e., photoaging). The oral mucosa, being protected from such environmental effects, shows few changes that can be unambiguously ascribed to aging. In some regions, there is a slight thinning of the epithelium with a concomitant flattening of the epithelial-connective tissue interface (64). Despite claims of a reduction in the rate of cell proliferation with age, there are no clear data to support this for human tissue, although there may be some increase in turnover time (65).

The limited information available on the permeability of oral mucosa indicates that there is a trend toward decreased permeability to water with age, which is statistically significant for floor-of-mouth mucosa from females (66). It is of interest to note that, in skin, where the morphologic changes with age are more marked than in oral mucosa, there have been a number of reports that demonstrate a significant decrease in permeability with age; Squier et al. (66) discussed the reasons for this.

Among the age changes evident in the lamina propria are those affecting the vascular system. Although there is some evidence for a reduction in the number of individual vessels with active flow (67), it is not known whether this reduction affects overall blood flow and perfusion. Systemic conditions encountered in the elderly that can affect the oral vasculature include diabetes and atherosclerosis (68,69). In a study of blood flow in atherosclerotic monkeys, Goodman and Squier (70) reported a 50% reduction in flow in the oral mucosa. However, given the ample blood supply to the oral tissues, it appears that perfusion is still sufficient to tissue viability, even in the presence of these vascular alterations (71).

RELATIONSHIP TO MUCOSAL INJURY IN CANCER

Anticancer therapy represents a significant challenge to the integrity of mucosal tissues. Chemotherapeutic agents and radiation limit proliferative ability so that the overlying epithelium becomes thin or ulcerated. This effect is first seen in the more rapidly proliferating tissues, such as gastrointestinal and oral lining mucosa, where atrophy and ulceration can represent a dose-limiting and potentially serious complication of treatment.

In the oral mucosa, lesions first appear on the soft palate, tongue, and cheeks; as they enlarge, they lead to extreme pain and dysphagia. As a consequence, there may be dehydration, a compromised nutritional status because of painful chewing, and a decreased quality of life.

The effect of cancer therapies is not limited to epithelia and will tend to lower cell proliferation and turnover in connective tissue; ionizing radiation has a direct effect on the tissue matrix leading to an increase in vascular permeability, tissue edema, and an infiltration of inflammatory cells. Damage to fibroblasts will result in cell loss and fibrosis; similarly, damage to blood vessels will lead to hypovascularity and tissue ischemia. Together, these changes will reduce the ability of the tissue to heal and resist infection. There may also be indirect effects, such as damage to the salivary glands, which will reduce salivary production and impair barrier efficiency, and a reduction in immunocompetence as a result of myeloablative therapy. This will increase the risk of local infection from oral organisms.

FUTURE RESEARCH DIRECTIONS

The treatment of mucosal injury in cancer patients has tended, in the past, to focus on palliation. Apart from effective combinations of antimicrobials, local anesthetics, and, possibly, anti-inflammatory agents, nonirritative vehicles that will coat the mucosa to enhance lubrication and provide some degree of occlusion so as to relieve acute symptoms have also been included. The discovery of potent agents that might protect or promote healing of the mucosal lining leads to the possibility of therapy rather than palliation. Most of the candidates are cytokines, with an effect on epithelial proliferation that has already been mentioned ("Cellular and Molecular Events in Differentiation in Oral and Esophageal Epithelium," *above*). While intuitively, it might be assumed that agents that increase epithelial proliferation would protect the epithelium during anticancer therapy, animal studies suggest the opposite effect—increasing proliferation sensitizes epithelial cells to the effects of chemotherapy and results in increased mucositis. This discovery has led to increased interest in cytokines that act by arresting epithelial cell division, thus sparing the cells from the effects of anticancer therapy. After release from arrest, there is rapid proliferation and repopulation of the tissue so as to restore normal mucosal function. Studies to identify other compounds with these effects and to characterize their behavior will be critical if management of mucosal injury is to progress from palliation to therapy.

The profound effect of cytokines on cell proliferation makes it essential that they be delivered locally to the mucosa, so as not to interfere with anticancer therapy. In practice, this demands topical application. To exert an effect after topical application, such compounds must pass across a surface permeability barrier (described in "Cellular and Molecular Events in Differentiation in Oral and Esophageal Epithelium," *above*) to reach the proliferative (basal) compartment of the epithelium. These requirements demand the maintenance of high local concentrations at the mucosal surface, so as to maintain a concentration gradient, and the presence of permeabilizers to ensure penetration of large molecules across the epithelial permeability barrier.

A major challenge in formulating topical agents for the oral cavity is the need for adhesion to the moist surface of the mucosa and the need to resist the flushing action of saliva. The use of bioadhesive gels reduces the frequency of application and the amount of drug administered and can also improve patient com-

pliance and acceptance. Optimizing the retention time of the drug is important in improving its clinical effectiveness. Finally, for the mucositis patient, the occlusion and lubrication of a bio-adhesive gel reduce the discomfort of the lesion.

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NOTE

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Protection Against Mucosal Injury By Growth Factors and Cytokines

Dawn Booth, Christopher S. Potten

This article provides an overview of published studies in which growth factors and cytokines were used to modify the sensitivity of intestinal stem cells to a dose of radiation. In these experiments, growth factors were used to manipulate the sensitivity of stem cells in the gastrointestinal tract to reduce the severity of gastrointestinal mucositis in cancer therapy patients. Transforming growth factor β 3, interleukin 11, and keratinocyte growth factor were used. All three agents, given according to appropriate protocols, can result in a threefold to fourfold increase in the number of intestinal stem cells that survive a dose of radiation therapy. This result was assessed by using the crypt microcolony assay of stem cell functional capacity. The changes in stem cell survival that were observed resulted in increased animal survival. This increased survival was taken as a surrogate for improvement in patient well-being. The severity of diarrhea, a marker of functional impairment, was concomitantly reduced. [J Natl Cancer Inst Monogr 2001;29:16–20]

Cancer therapy involves a fine balance between the use of a large enough dose of a drug to kill tumor cells and the prevention of damage to the normal tissues of the body, notably the oral mucosa, the gastrointestinal tract, and the hematopoietic system. Damage caused during cancer therapy to the epithelial layer lining the mouth causes the lining to become depleted and ulceration of the mouth to occur (oral mucositis). This makes it difficult for the patient to eat, swallow, or speak and causes pain and susceptibility to infection. A combination of patient discomfort and possible infection can lead to treatment delay or dose reduction, which can result in a less favorable outcome for the patient. Another major dose-limiting tissue is the gastrointestinal tract, whose rapid cell cycle makes it more susceptible to the effects of cytotoxic exposure and to a rapid expression of damage. This damage can be seen in the small intestine within a few days and within a slightly longer period of time in the large bowel. This article will concentrate on the specific problems associated with damage to the normal small intestine.

The small intestine is a constantly renewing tissue, continuously replacing cells that are lost in the lumen of the intestine. This renewal is achieved by the production of new cells in the crypts by the stem cells and their progeny, arranged in an amplifying transit lineage of six to eight generations. Under steady-state situations, there are thought to be between four and 16 actual stem cells that are located near the base of the crypt. However, if the system is damaged (e.g., by radiation) an acute response occurs, causing some cells in the lower region of the crypt, possibly the stem cells, to die via apoptosis. The stem cells, probably along with some early transit generation cells, will recognize the damage and undergo rapid cell division to regenerate the crypt and, hence, the tissue by clonal growth. The number of clonogenic cells per crypt is thought to depend on the level of damage induced, but it could be as many as 30 cells per

crypt (1). Some crypts will become reproductively sterile, will be unable to initiate this regeneration response, and will disappear within approximately 48 hours.

The time sequence for this response is as follows (Fig. 1). In the first 2–3 days following a dose of radiation (e.g., a 14-Gy dose), some crypts can be seen to be regenerating (forming microcolonies) alongside other crypts that have been reproductively sterilized. On day 4 following radiation therapy, those crypts that have survived the radiation damage will be approximately 1.5 to two times bigger than a normal crypt, and the sterilized crypts will have disappeared. These large, regenerating crypts will then start to split or bud into several crypts (2). Approximately 14 days after irradiation, the regenerating crypts and foci will have grown visible to the naked eye as macrocolonies (3,4). In certain cases, budding will continue until the whole of the intestine has been regenerated and the normal architecture restored.

There are advantages in protecting the clonogenic stem cells from damage, since they are the key to the survival of an individual crypt. The number of crypts that survive following cytotoxic damage determines how intact the intestinal mucosa is and, hence, how well an animal or a patient can survive the damage. The number of surviving crypts plays a pivotal role in the competitive race between depopulation and ulceration and regeneration. Alternatively, a stimulatory factor given before cytotoxic exposure could increase the number of stem cells per crypt that are subjected to the cytotoxic insult. A possible method of protecting the clonogenic stem cells might be to manipulate them by using growth factors. This could be achieved by using a growth factor that will take the stem cells out of the cycle, making them more resistant to the cytotoxic damage, and ensuring the survival of more stem cells capable of regenerating any damaged tissue. Finally, giving, after cytotoxic insult, a growth factor that could increase the rate of proliferation or initiate regeneration earlier may help in speeding up the regeneration process. A combination of various protocols could give maximum protection for the epithelium.

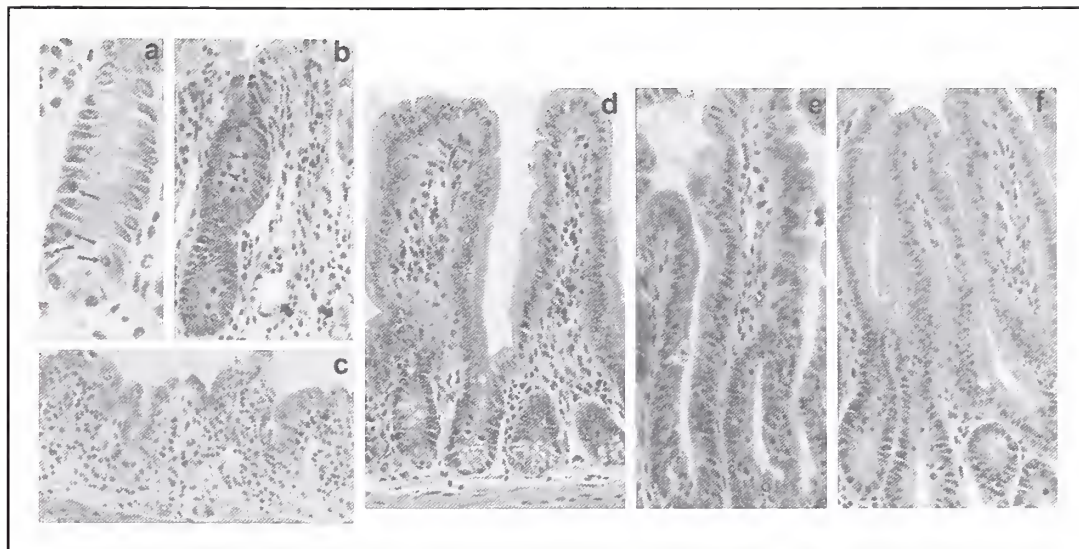
Various experiments to manipulate clonogenic stem cells to protect against cytotoxic damage have been carried out, but this review will outline work that has used transforming growth factor β 3 (TGF- β 3), interleukin 11 (IL-11), and keratinocyte growth factor (KGF), using 10–12-week-old (C57BL/6 \times DBA/2) F_1 (BDF $_1$) mice. All experiments were performed within the regulations of the U.K. Scientific Procedures Act (1986).

Affiliation of authors: Cancer Research Campaign Epithelial Biology Group, Paterson Institute for Cancer Research, Christie Hospital, Manchester, U.K.

Correspondence to: C. S. Potten, B.Sc., M.Sc., Ph.D., D.Sc., Epistem Ltd., Incubator Building, Grafton St., Manchester M13 9XX, U.K. (e-mail: cpotten@epistem.co.uk).

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Fig. 1. Radiation effects on small intestine. (a) (Original magnification $\times 100$). The earliest response is an increase in apoptosis 4.5 hours following 8-Gy irradiation. **Arrows** indicate apoptotic bodies. (b) (Original magnification $\times 50$). A selected area of intestine demonstrating a single large regenerating crypt 4 days after 14-Gy irradiation. Vincristine helps identification by arresting cells in metaphase. **Arrowheads** = mitosis. **Larger arrows** indicate two reproductively sterilised crypts that are dying and disappearing. (c) (Original magnification $\times 40$). Day 6 following 14-Gy irradiation. Many areas of intestine are completely devoid of crypts and villi with only a few epithelial cells remaining.



(d) (Original magnification $\times 40$.) Section through a normal (control) small intestine showing crypts and villi. (e) (Original magnification $\times 40$). By day 8 following 14-Gy irradiation the few regenerating crypts (see Fig. 1, c) have split (budded) and are starting to form new crypts and villi. (f) (Original magnification $\times 40$.) By day 30 following 14-Gy irradiation the epithelium in the surviving animals is restored to its normal small intestinal architecture, as seen in Fig. 1, d.

TRANSFORMING GROWTH FACTOR $\beta 3$

TGF- $\beta 3$ is a known inhibitor of some epithelial cell proliferation (5,6) by preventing cell cycle progression and accumulating cells in G_1 or G_0 . For clonogenic stem cells in the intestine, this might render them more resistant to the cytotoxic damage, leaving more clonogenic stem cells to start the regeneration process.

To assess the capacity of TGF- $\beta 3$ to protect the intestine from radiation damage, we used the crypt microcolony assay as described by Withers and Elkind (7). This is an accepted test for crypt stem cell functional capacity. A standard dose of 2.5 mg TGF- $\beta 3$ was administered intraperitoneally to male BDF₁ mice 24, 8, and 4 hours before and then once immediately after irradiation. Animals were culled and samples of small intestine were taken 4 days after irradiation. The number of surviving crypts per circumference (a unit length) was counted for 10 circumferences per mouse. Data were analysed by using the DRFIT program (8), which allows curves to be fitted and tests the statistical difference between treated and vehicle control groups by using a variance-ratio F test. As shown in Fig. 2, a, four injections of TGF- $\beta 3$ administered once immediately after and again at 4, 8, and at 24 hours before radiation therapy compared with the vehicle control shows a statistically significant shift to the right of the treated curve (9) ($P < .001$). This indicates that there are four times more clonogenic stem cells surviving at 15 Gy in the TGF- $\beta 3$ -treated group than in the control group. It is interesting that if an additional dose of TGF- $\beta 3$ was administered 4 hours after irradiation (-4, -8, -24, 0, and +4 hours), then the level of protection afforded is reduced (data not shown). TGF- $\beta 3$ administered intraperitoneally after radiation therapy caused crypts to become sensitized, with only approximately one third of the crypts surviving, in comparison with the vehicle control (data not shown). This finding demonstrates the importance of the use of a growth factor in the correct protocol.

These studies demonstrated that the small intestinal clonogenic stem cells can be protected from radiation damage. How does this finding relate, however, to the patient's well-being in

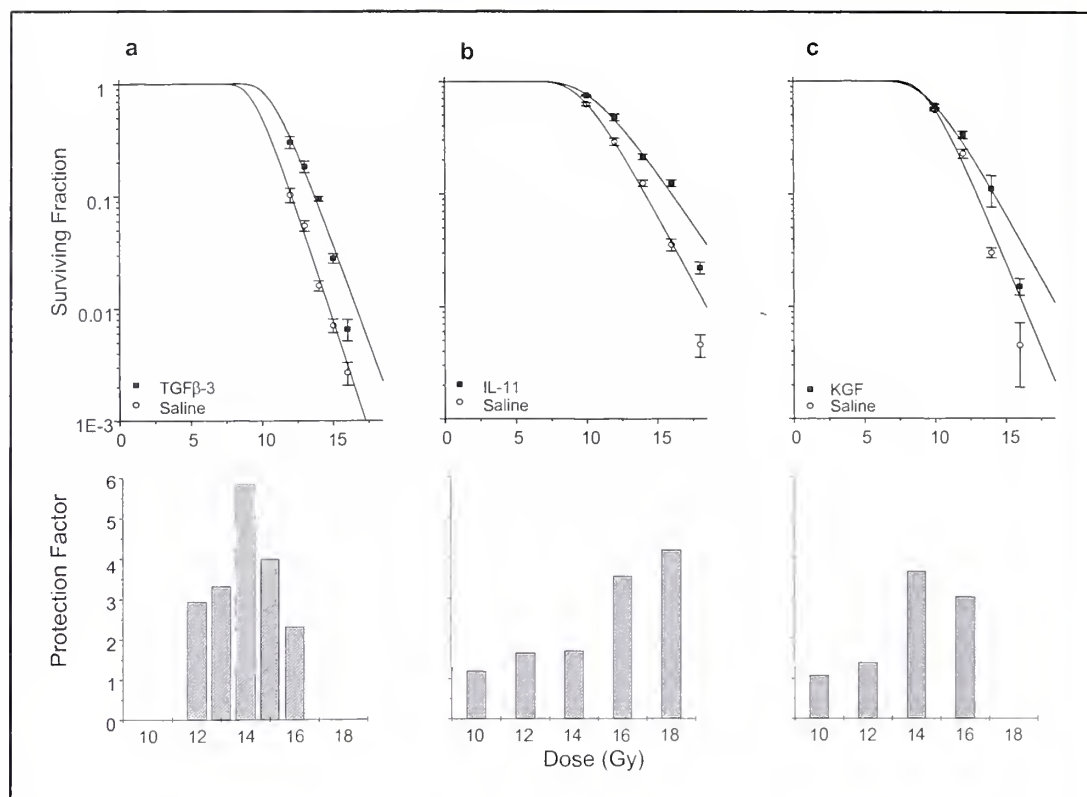
cancer therapy? To answer this question, we conducted a series of experiments to study animal survival over time after a dose of radiation. Groups of 20 animals were pretreated with TGF- $\beta 3$ or vehicle, then irradiated with a 15.8-Gy x-ray and observed for 30 days. Animals had their heads, thoraxes, and forelimbs shielded to reduce the complications of oral and hematopoietic damage. Almost 100% of animals pretreated with TGF- $\beta 3$ survived to 30 days, but only approximately 35% of the vehicle control animals survived (10) (Fig. 3, a). During the 30 days postirradiation, animals were checked twice daily for diarrhea (verified by evidence of wet feces on the fur of the anal region). The duration of diarrhea can be seen on individual animal "life-lines" as shown in Fig. 4. Administration of TGF- $\beta 3$ before the radiation dose reduces the total number of days during which diarrhea was recorded, suggesting not only an improvement in life expectancy within this experiment but also an improvement in quality of life and a reduction in diarrhea, a surrogate marker of intestinal dysfunction (10). The histology of the intestine of the animals surviving to 30 days is indistinguishable from that of control subjects (Fig. 1). This indicates truly remarkable regenerative capacity, especially when the appearance of the intestine at days 3-5 (Fig. 1) is considered.

INTERLEUKIN 11

A pleiotrophic cytokine that affects many different systems, recombinant human IL-11 was originally isolated and cloned from an immortalized primate bone marrow stromal cell line (11). Similar experiments to the TGF- $\beta 3$ study were carried out by using IL-11.

A number of protocols were tested with use of the crypt microcolony assay, and these were carried out as follows: protocol A: IL-11 administered subcutaneously at 9 AM and at 9 PM, for a total of five injections before irradiation; protocol B: IL-11 administered subcutaneously at 9 AM and at 9 PM, for a total of seven injections postirradiation; and protocol C: IL-11 administered subcutaneously at 9 AM and at 9 PM, for a total of three injections prior to irradiation and six injections following irradiation.

Fig. 2. Intestinal crypt survival curves comparing treated groups against respective vehicle controls. All vehicle groups are represented as **open circles** and all treated groups as **closed symbols**. (a) Animals were given vehicle or 2.5 mg transforming growth factor $\beta 3$ (TGF- $\beta 3$) intraperitoneally at 24, 8, and 4 hours before irradiation and once immediately after irradiation. Values defining the TGF- $\beta 3$ survival curve are (mean \pm SE) $D_0 = 1.26 \pm 0.10$, $N = 5664 \pm 4698$ and the values for the vehicle curve are $D_0 = 1.12 \pm 0.83$, $N = 5294 \pm 4492$. There is a statistically significant difference between the two curves ($P < .001$). D_0 is the mean lethal dose, the reciprocal of the slope on the exponential position of the curve—it is a measure of the radiosensitivity; N is the back extrapolate to zero dose of the exponential portion of the curve and a measure of the size of the shoulder. (b) Animals were given vehicle or 2.5 mg interleukin 11 (IL-11) subcutaneously 1 day before, at the time of irradiation, and continuously throughout the postirradiation regeneration phase. All injections were given at 9:00 AM and 9:00 PM, for a total of three injections before irradiation and six injections after irradiation. Values for the IL-11 survival curves are (mean \pm SE) $D_0 = 2.30 \pm 0.94$, $N = 110.5 \pm 23.7$, and the values for the vehicle group are $D_0 = 1.84 \pm 0.67$, $N = 232.5 \pm 52.9$. There is a significant difference between the two curves ($P < .001$). (c) Animals were given vehicle or 12.5 mg keratinocyte growth factor (KGF) subcutaneously once a day for 3 days before irradiation. Values for the KGF curve are $D_0 = 1.91 \pm 0.12$, and $N = 173 \pm 65$, and the values for the vehicle control curve are $D_0 = 1.43 \pm 0.06$, and $N = 886 \pm 294$. There is a statistically significant difference between the two curves ($P < .001$).



In all cases, IL-11 protected the clonogenic stem cells from radiation damage, but to varying degrees. Protocol A gave good protection with a statistical significance of $P < .001$, and protocol B was only modestly protective ($P = .003$), while the best protection was afforded by administering IL-11 subcutaneously both before and after radiation therapy (protocol C; $P < .001$) (Fig. 2, b) (12), when up to 3.5 times more crypts survived a dose of 16-Gy irradiation in the IL-11 treated groups.

Studies examining IL-11 and animal survival over time after radiation therapy did not take into consideration the damage caused to the hematopoietic system. Protocols A and C were carried out with a dose of 12-Gy x-ray irradiation. Animals were observed over the following 30 days. As a result of damage to the hematopoietic and gastrointestinal systems, all of the animals treated with a dose of 12-Gy irradiation died during the first 11 days; however, the IL-11-treated groups survived an average of 2 days longer than the controls (Fig. 3, b) (13).

It is unclear by what mechanisms IL-11 protects against cytotoxic damage, but IL-11 given before radiation therapy alters the sensitivity of clonogenic stem cells, making them more resistant; hence, more of them survive. It is uncertain whether IL-11 alters cell cycle progression or has an indirect effect by altering other growth factors. IL-11 protects best when it is given both before and after irradiation, and it may act via different mechanisms before and after irradiation. IL-11 is known to synergize with other factors [e.g., stem cell factor and steel factor (14)] and also may enhance the effects of IL-3 (15).

KERATINOCYTE GROWTH FACTOR

KGF was identified originally as a factor that stimulated epithelial cells *in vitro* (16). Further studies demonstrated that it also stimulates both proliferation and differentiation in a number of epithelial cell types when given to healthy animals. This is very noticeable in the gastrointestinal tract (17). It has been observed that administration of KGF has trophic effects on the gastrointestinal tract, increasing crypt depth and villus length (18). KGF also has a particularly pronounced trophic effect on oral mucosa (19).

Crypt survival studies with the use of KGF as a protective agent against radiation damage have shown that KGF given for 3 days before irradiation showed a statistically significant protective effect over a range of doses ($P < .001$) (Fig. 2, c), with three times more crypts surviving a dose of 16-Gy irradiation in the KGF treated group and 3.5 times more crypts surviving after a dose of 14-Gy irradiation than controls (vehicle treated) (18). Use of KGF postirradiation was not found to improve crypt survival.

Animal survival studies have also been performed with KGF and 12 Gy of radiation by using postirradiation bone marrow transplantation to protect the hematopoietic system (Fig. 3, c). This experiment showed that 90% of mice pretreated with KGF before receiving 12 Gy of irradiation and a bone marrow transplant survived a 16-day observation period, whereas mice that received only 12 Gy of irradiation and a bone marrow transplant all died during the first 8 days.

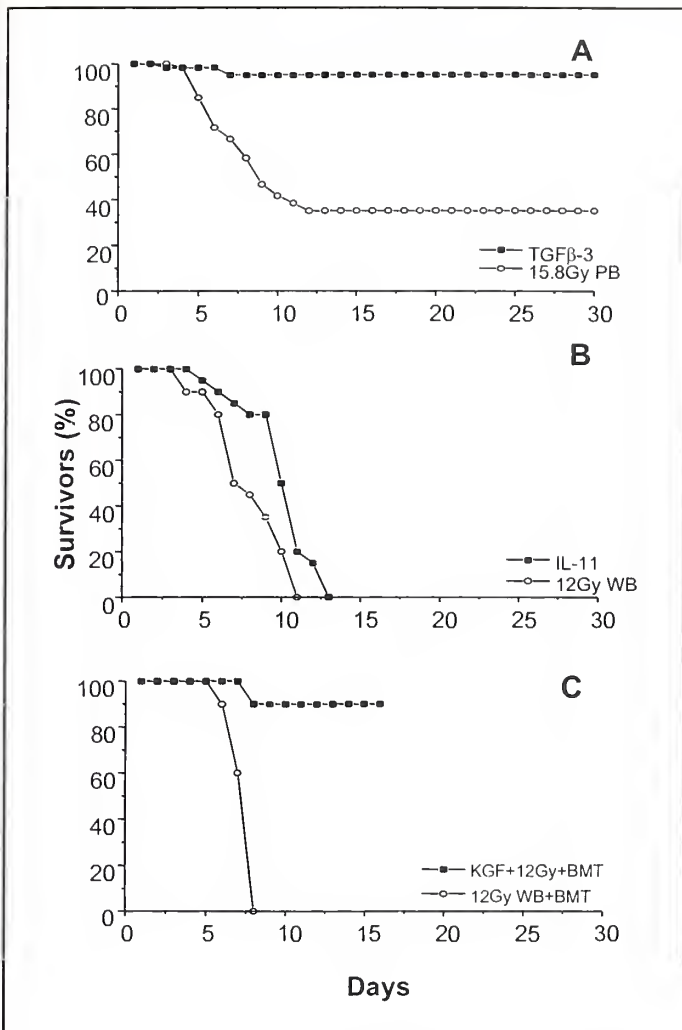


Fig. 3. Survival time of animals exposed to 12-Gy (experiment with interleukin-11 [IL-11] or 15.8-Gy (experiment with transforming growth factor β 3 [TGF- β 3]) x-rays delivered whole body (IL-11) or abdomen only (TGF- β 3)). For the keratinocyte growth factor (KGF) experiment, 12-Gy Cs¹³⁷ was delivered whole body, followed by a bone marrow transplant. All vehicle groups are represented as **open symbols**, and all treated groups are represented as **closed symbols**. **A)** Animals were given vehicle or 2.5 mg TGF- β 3 24, 8, and 4 hours before irradiation and once immediately after irradiation. **B)** Animals were given vehicle or 2.5 mg IL-11 subcutaneously 1 day before irradiation, at the time of irradiation, and continuously throughout the postirradiation regeneration phase. All injections were given at 9:00 AM and 9:00 PM. **C)** Animals were given vehicle or 5 mg/kg KGF per day subcutaneously on days 2, 1, and 0; that is, before and at the time of (day 0) radiation. PB = partial body, WB = whole body, BMT = bone marrow transplant.

It seems somewhat puzzling that the best protocol for protection is to give KGF before cytotoxic insult when it is known that KGF is a stimulator. The mechanism by which KGF protects the intestinal system is unknown and could be multifactorial. However, KGF has been shown to have trophic effects on the gastrointestinal tract, increasing crypt size. This may suggest that KGF protects the crypts by proportionally increasing the number of clonogenic stem cells per crypt (18). Indeed, cell proliferation studies have clearly indicated increased stem cell proliferation following KGF treatment, as well as (rather surprisingly) a reduction in transit cell proliferation (20). Stimulatory factors, such as KGF and, possibly, IL-11, may also act as cell survival factors that prevent apoptosis.

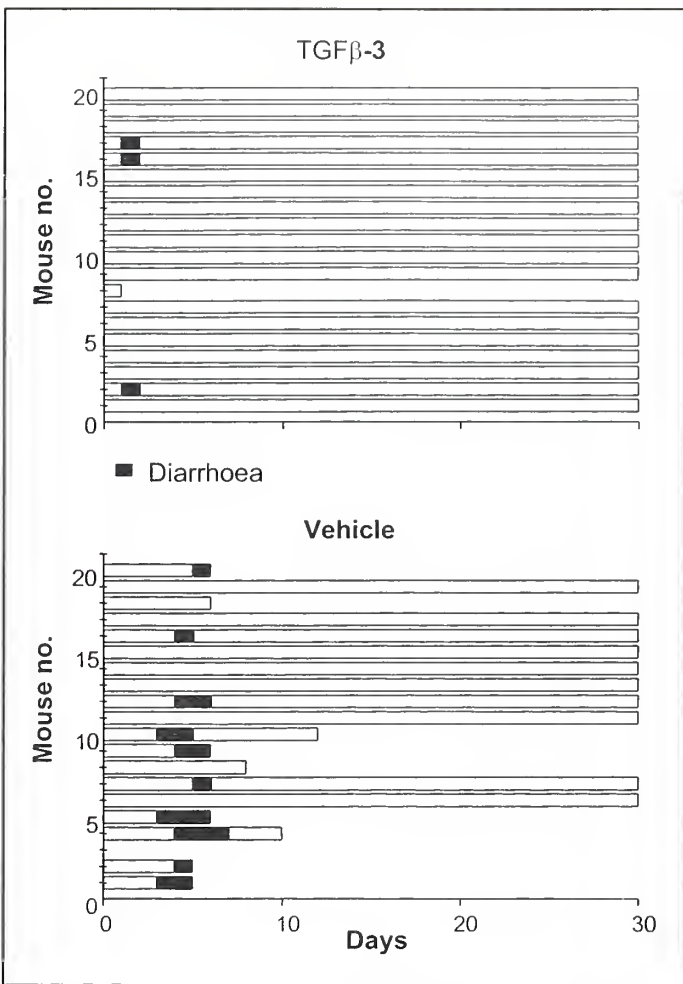


Fig. 4. Each line represents the life of an animal through a survival experiment, with the **black boxes** indicating when diarrhea was observed and the **termination of a line** indicating when an animal died. This is taken from an experiment where a dose of 15.3 Gy partial body x-ray was delivered to the abdomen and the mice were pretreated with transforming growth factor β 3 (**top panel**) or vehicle (**lower panel**).

KGF has also been shown to increase the number of goblet cells in the gastrointestinal tract (17). Goblet cells produce mucins that act as a barrier between the epithelium and the luminal and trefoil proteins that act to defend the gut and can also aid in its repair.

One difficulty in making comparisons between the effects of these different agents is that different injection regimens have been used, as have, in some cases, different radiation doses and delivery protocols. However, it is very clear that the sensitivity of the potential clonogenic stem cells in the small intestine of the mouse can be experimentally manipulated by exogenous growth factors or cytokines in an advantageous manner. Such manipulations have been shown to afford overall radioprotection to an animal, and this protection manifests itself in potentially dramatic changes in animal survival and well-being.

CONCLUSIONS

These studies show that statistically significant and, in some cases, dramatic reductions in mucositis can be effected by appropriate manipulation of stem cell sensitivity with the use of growth factors and cytokines. The results presented here are

preliminary; extensive additional studies are required to determine the most effective doses and delivery protocols. Many more growth factors and cytokines should be tested, together with combined and sequential use of different factors. With the identification of intestinal-specific regulatory factors, it would seem likely that the sensitivity of the critical stem cells might be even more effectively manipulated. This would reduce the severity of gastrointestinal mucositis even further, improving the quality of life of cancer therapy patients and possibly allowing for dose escalation and improvement in cure rates.

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Transgenic Mouse Model of Intestine-Specific Mucosal Injury and Repair

Leo Lefrançois, Vaiva Vezys

Most studies of injury and repair to mucosal tissue have used nonspecific mediators to induce injury. Damage to the mucosal epithelium resulting from chemical or radiation treatment associated with cancer therapy may fall into this category of injury. When such treatments are applied, it is generally not possible to predict or control the extent of possible injury. This fact makes analysis of inductive and reparative processes difficult. In addition, the role of the immune system in the etiology and subsequent healing of mucosal tissue following cancer therapy with or without bone marrow transplantation remains unclear. To study tissue- and antigen-specific immune damage of intestinal mucosal tissue, we generated transgenic mice that express a nominal antigen exclusively in intestinal epithelial cells. The transfer of antigen-specific CD8 T cells with concomitant virus infection resulted in the destruction of intestinal epithelial cells and disease. The destructive phase in some cases was followed by complete recovery and tolerance induction. This model will provide a system that can be regulated for analysis of the mediators of mucosa-specific tissue damage and repair. [J Natl Cancer Inst Monogr 2001;29:21-5]

The intestinal mucosal immune system is composed of inductive and effector sites. Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) serve as sites for antigen presentation and primary activation of naive T and B cells. After activation in these sites, some lymphocytes then migrate to tertiary tissue sites, including the epithelium, the lamina propria (LP), and the effector sites of the intestinal mucosa (1,2). Numerous activated CD4 and CD8 T cells, as well as B and plasma cells, can be found within the LP (3-7). Plasma cells in the LP mainly produce immunoglobulin A, which is subsequently actively transcytosed across the epithelium by a receptor-mediated process (8). The majority of this antibody is presumed to be specific for bacterial antigens. Similarly, in normal situations, the activated and memory T cells residing in the LP and the epithelium may have been initially primed by antigens derived from normal flora. Indeed, in germ-free mice, the intestinal immune system is poorly developed and contains severely reduced numbers of lymphocytes with reduced effector function (6,9). Thus, colonization of the gut with normal flora is, in part, responsible for the formation of the mucosal immune system tissue. This process is, in effect, a symbiotic relationship between bacteria and the host in which the latter gains considerable benefit through the formation of a normal mucosal immune system poised to respond to pathogenic insult.

The intestinal epithelium is home to a substantial population of T lymphocytes termed "intraepithelial lymphocytes" (IELs). IELs are a complex population of cells that contain several lymphocyte subsets (10). Most small intestinal IELs express CD8, while large intestinal IELs contain larger populations of CD4 and CD4-8-T-cell receptor (TCR) $\alpha\beta$ subsets (11,12). TCR us-

age is also distinct for IELs as compared with lymphocytes in secondary lymphoid tissue, such as that found in the spleen and lymph nodes. Small intestinal IELs in most species examined contain an appreciable population of cells that express TCR $\gamma\delta$ [$\geq 50\%$ in some mouse strains (13-15)]. This enigmatic population of lymphocytes has been proposed to be involved in early responses to bacterial infection, perhaps through recognition of antigens presented by nonclassic major histocompatibility complex (MHC) molecules. The precise function of TCR $\gamma\delta$ IELs remains elusive, although these cells have been shown to produce keratinocyte growth factor (KGF) when activated, suggesting that they may play a role in homeostasis or repair of the intestinal epithelium (16).

Mucosal epithelial cells are far from being simply a quiescent physical barrier. Rather, intestinal epithelial cells (IECs) are active participants in innate as well as adaptive immune responses (17,18). IECs produce, as well as respond to, a variety of cytokines, including factors involved in lymphocyte development, such as stem-cell factor and interleukin 7 (19,20). IECs also produce chemokines that are likely essential for mucosal lymphoid organ formation and for mounting effective immune responses (21-23). In inflammatory bowel disease, IECs can become targets of a dysregulated immune system (24). It remains unclear whether IECs are victims of direct antigen-specific immune destruction or whether inflammatory cytokines induce damage in a bystander-like fashion. To determine the consequences of antigen-specific T-cell interactions with IECs, we established the model presented here.

MATERIALS AND METHODS

Mice. C57BL/6J (Ly5.1) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. The OT-I mouse line (25) was from W. R. Heath (Walter and Eliza Hall Institute, Parkville, Australia) and F. Carbone (Monash Medical School, Prahan, Victoria, Australia) and was maintained as a C57BL/6-Ly5.2 line on a RAG-/- background. Intestinal fatty acid-binding protein promoter-truncated ovalbumin (IFABP-tOVA) transgenic mice were created by use of a construct containing the long form of the IFABP promoter (nucleotides -1178 to +28; a gift of J. I. Gordon, Washington University School of Medicine, St. Louis, MO) (26,27), tOVA cDNA (encoding amino acids 138-386), which does not include the signal sequence so the protein remains cytosolic (28), and human growth hormone (hGH)

Affiliation of authors: Division of Rheumatic Diseases, Department of Medicine, University of Connecticut Health Center, Farmington.

Correspondence to: Leo Lefrançois, Ph.D., Division of Rheumatic Diseases, Department of Medicine, University of Connecticut Health Center, MC1310, 263 Farmington Ave., Farmington, CT 06030 (e-mail: llefranc@neuron.uhc.edu).

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(nucleotides 498–2652; from R. M. Perlmutter, University of Washington, Seattle) (29). An *SalI* fragment containing these elements was microinjected into C57BL/6-Ly5.1 fertilized eggs by the Transgenic Animal Facility at the University of Connecticut Health Center. To detect the transgene, genomic DNA was analyzed by polymerase chain reaction to identify a 608-base-pair band by using an IFABP-specific 5' primer (5'-GCCATC-ACACTTGACCCTAA-3') and an OVA-specific 3' primer (5'-TCAGGCAACAGCACCAACAT-3'). Mice were kept in specific pathogen-free housing and were analyzed between 8 and 10 weeks of age.

RNA analysis. Total RNA from the indicated tissues was isolated by cell lysis with guanidine isothiocyanate followed by 16 hours of centrifugation at 22 °C over a cesium chloride cushion (30). Purification of poly (A) RNA was accomplished by using a Poly(A) Quik messenger RNA (mRNA) Isolation Kit from Stratagene (La Jolla, CA), according to the manufacturer's instructions. One microgram of poly (A) RNA was dot blotted onto a nylon membrane, which was then hybridized with a ³²P-labeled OVA cDNA fragment. The blot was stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase-specific cDNA probe to allow for mRNA quantification. A Molecular Dynamics (Sunnyvale, CA) PhosphorImager was used to quantitate hybridization.

Isolation of lymphocyte populations and adoptive transfer of OVA-specific CD8 T cells. IEL and LP cells were isolated as described previously (12,13). Lymph nodes (LN) and spleens were removed and single-cell suspensions were prepared. Peripheral LN included brachial, axillary, and superficial inguinal lymph nodes. The resulting preparation was filtered through Nitex, and the filtrate was centrifuged at 200g for 5 minutes at 4 °C to pellet the cells. For adoptive transfer, 5 × 10⁵ OT-I/RAG-/-/Ly5.2 pooled LN cells were injected intravenously into Ly5.1 B6 or transgenic hosts. Where indicated, mice were infected 24 hours later by intravenous (IV) injection of 1 × 10⁶ plaque-forming units (pfu) vesicular stomatitis virus (VSV)-encoding OVA (31).

Detection of antigen-specific CD8 T cells with MHC tetramers. Mice were infected by injection of 1 × 10⁶ pfu of VSV-OVA. Six days later, lymphocytes were isolated and VSV nucleoprotein (N)-specific or OVA-specific CD8 T cells were detected by using H-2K^b tetramers containing the N protein-derived peptide RGYVYQGL (32) or the OVA-derived peptide SIINFELK (33). Peptides were purchased from Research Genetics, Huntsville, AL. Tetramers were produced essentially as described previously (34,35). In brief, H-2K^b containing the BirA-dependent biotinylation substrate sequence (the construct was provided by J. Altman, Emory University, Atlanta, GA) was folded in the presence of human β2-microglobulin and the N or OVA peptide. Biotinylation was performed with biotin-protein ligase (Avidity, Denver, CO). Tetramers were then produced from biotinylated high-pressure liquid chromatography-purified monomers by the addition of streptavidin-allophycocyanin (APC) (Molecular Probes, Eugene, OR).

Flow cytometric analysis. Lymphocytes were resuspended in 0.2% phosphate-buffered saline (PBS), 0.1% bovine serum albumin (BSA), and NaN₃ at a concentration of 1 × 10⁶ to 1 × 10⁷ cells/mL followed by incubation at 4 °C for 30 minutes with 100 μL of properly diluted monoclonal antibody (MAb). The MAbs either were directly labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), Cy5, APC or were biotinyl-

ated. For the latter, avidin-PE-Cy7 (Caltag Laboratories, Burlingame, CA) was used as a secondary reagent for detection. For tetramer staining, cells were first reacted with PE-labeled anti-CD8 (Caltag Laboratories) and FITC-labeled anti-CD11a at 4 °C followed by staining for 1 hour at room temperature with APC-coupled MHC tetramers. After staining, the cells were washed twice with PBS/BSA/NaN₃ and fixed in 3% paraformaldehyde in PBS. Relative fluorescence intensities were then measured with a FACSCalibur (Becton-Dickinson, San Jose, CA). Data were analyzed by using WinMDI software (J. Trotter, Scripps Clinic, La Jolla, CA).

Histologic analysis. Duodenum, jejunum, and ileum from experimental animals were fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA). Paraffin-embedded tissue was sectioned and then stained with hematoxylin-eosin. All images were magnified × 200.

RESULTS

With the goal of testing the impact of T-cell reactivity with an IEC-specific antigen, we generated transgenic mice expressing a chicken tOVA gene under control of an IEC-specific promoter (Fig. 1). The IFABP directs protein expression to mature enterocytes but not to crypt epithelial cells (26,27). In addition, IFABP is expressed primarily in the small intestine and is expressed weakly or not at all in the stomach or the large bowel. The hGH gene that contains introns and exons was used to provide signals for the poly (A) addition and to increase *in vivo* transgene expression (29). When expressed, the OVA lacks a signal sequence, so that the protein remains cytosolic (28). Because of this, IECs should effectively process and present OVA-derived peptides in the context of MHC class I. The mouse strain used for transgenic production was C57BL/6J, an H-2^b haplotype strain. The OVA contains an eight amino acid peptide, SIINFELK, which has been shown to bind to H-2K^b (33). Thus, MHC class I-restricted CD8 T cells of the appropriate specificity should recognize IECs expressing the tOVA.

We examined the OVA mRNA levels in two lines of IFABP-tOVA transgenic mice. There was a striking difference in mRNA levels between the two lines, with the 232-4 line expressing approximately 10-fold more mRNA than the 232-6 line. The expression patterns were similar in the two lines, with the highest concentrations of OVA mRNA present in the ileum. Fig. 2 depicts the relative mRNA levels detected in sections of the intestine of IFABP-tOVA mice. We were unable to detect OVA by immunohistochemistry or western blotting, suggesting that low levels of protein were expressed or the truncated protein was rapidly degraded in the cytoplasm of IECs. We first tested whether CD8 T cells of the transgenic mice were tolerant to

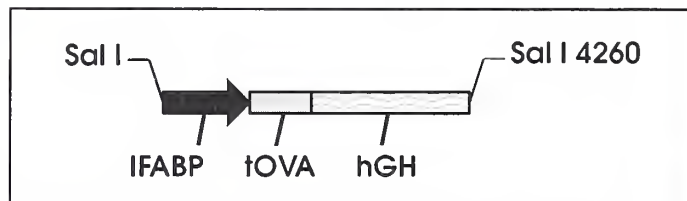


Fig. 1. Construct used in generation of intestinal fatty acid-binding protein promoter-truncated ovalbumin (IFABP-tOVA) transgenic mice. The *SAL I* fragment used for production of C57BL/6J transgenic mice contained the long form of the IFABP promoter, tOVA complementary DNA (cDNA)-encoding amino acids 138–336, and the human growth hormone complementary DNA.

Expression of the iFABP-tOVA Transgene

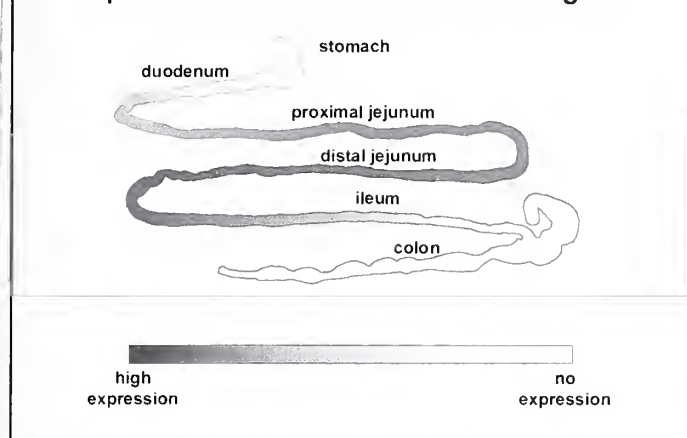


Fig. 2. Depiction of the relative concentration of ovalbumin (OVA) messenger RNA in 232 mice along the length of the intestine. The figure is based on data from dot blot analysis of poly(A) RNA from duodenum, proximal jejunum, distal jejunum, and ileum of intestinal fatty acid binding protein promoter-truncated OVA mice by using OVA complementary DNA as a probe.

OVA. This test was performed by infecting the mice with a recombinant virus containing the OVA gene (VSV-OVA). In normal mice, a robust OVA-specific CD8 T-cell response can be visualized by use of MHC class I tetramers that contain the SIINFEKL peptide. In contrast, few if any OVA-specific CD8 T cells could be found after VSV-OVA infection of IFABP-tOVA mice (36). This result indicated that IEC-expressed antigen had gained access to the systemic immune system. The mechanism by which this occurred is unknown but may be mediated by dendritic cells carrying IEC-derived proteins to the draining lymph nodes and beyond (37). At that point, cross tolerance would be induced to this "self-antigen" via interaction of T cells with dendritic cells that had not been activated by inflammatory signals (see Fig. 4). Further experiments will be necessary to delineate this important pathway for maintenance of self-tolerance to gut-specific protein.

Since the CD8 T-cell compartment of IFABP-tOVA transgenic mice was apparently tolerant to OVA, we were unable to test the reactivity of endogenous T cells with IECs. To circumvent this problem, we used an adoptive transfer system in which naive, OVA-specific TCR transgenic CD8 T cells (called OT-I) were transferred to unmanipulated IFABP-tOVA mice (Fig. 3). The transferred cells are trackable by virtue of differences between host and donor Ly5 alleles. Thus, a monoclonal antibody specific for Ly5.2 (expressed by the donor CD8 T cells but not by host cells) can be used in flow cytometry or immunohistochemistry to detect the OVA-specific CD8 T cells after transfer to the transgenic mice (31). Small numbers (5×10^5) of OT-I cells were injected by the IV route into host transgenic mice; their presence was detected 4 and 5 days later in secondary lymphoid tissues and in intestinal mucosal effector sites (LP and epithelial lymphocytes, respectively). Four days after transfer to the low antigen-expressing line 232-6, few OT-I cells were present in peripheral lymph nodes (PLNs), LP, or IELs (Table 1). In contrast, a large expansion of OT-I cells had occurred at this time point in PP and MLN. By day 5 after transfer, the MLN and PP OT-I populations had declined, whereas significant numbers of OT-I cells were now present in LP and IELs. Four days after

MODEL SYSTEM FOR ANALYSIS OF T CELL REACTIVITY WITH INTESTINE-SPECIFIC ANTIGEN

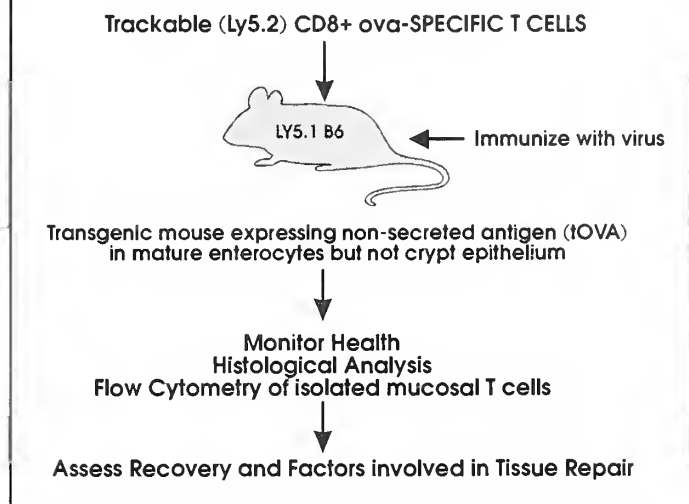


Fig. 3. Model system for analysis of immune-mediated intestinal epithelial cell injury.

Table 1. Mucosa-specific expansion of antigen-specific CD8 T cells adoptively transferred to IFABP-tOVA transgenic mice*

Mouse line	Tissue				
	PLN	MLN	PP	LP	IELs
232-6					
Day 4	1.1 ± 0.2	11.3 ± 3.5	44.3 ± 6.5	1.8 ± 0.6	1.1 ± 0.3
Day 5	0.7 ± 0.3	3.8 ± 0.8	30 ± 1.0	11.4 ± 2.7	8.8 ± 1.2
232-4					
Day 4	5.2 ± 0.7	28.7 ± 3.5	49.3 ± 9.1	11.6 ± 1.9	11.7 ± 1.3
Day 5	2.6 ± 0.4	15.2 ± 1.9	57.9 ± 4.8	50.7 ± 9.6	60.9 ± 7.4

*PLN = peripheral lymph nodes; MLN = mesenteric lymph nodes; PP = Peyer's patch; LP = lamina propria; IELs = intraepithelial lymphocytes. 5×10^5 Ly5.2 OT-I-RAG-/- T cells were transferred to Ly5.1 IFABP-tOVA transgenic mice (232-6 or 232-4). Four or five days later lymphocytes were isolated from the indicated tissues and the presence of CD8+ OT-I cells was determined by fluorescence flow cytometry. Values indicate means and standard errors of at least six determinations per tissue.

transfer of OT-I cells to the high antigen-expressing line 232-4, many donor cells were found in MLN and PP. Unlike in the 232-6 mice, OT-I cells were also found in the LP and IELs at this time point. By day 5 after transfer to 232-4 mice, a massive expansion of OT-I cells had occurred in the LP and IEL compartments. Overall, these results suggested that IEC-derived OVA or OVA fragments are being transferred to PP and MLN, where antigen presentation to T cells can occur. The extent of activation is dependent on the antigen levels, since less activation occurred in the 232-6 (low antigen) compared with the high antigen 232-4 mice. These findings also indicated that activated CD8 T cells did not enter the intestinal mucosa until after activation in PP or MLN, since there were substantial numbers of activated OT-I cells in PP and MLN at least 1 day before their appearance in the mucosa. These data are in agreement with our previous study (2) showing that $\alpha 4 \beta 7$ integrins are important for migration of activated OT-I cells into the intestinal mucosa.

Despite the fact that large numbers of antigen-specific CD8 T cells entered the mucosa and apparently responded to antigen expressed by IEC, no overt damage to intestinal tissue was observed by histologic analysis. OT-I cells isolated from the epithelium of these animals exhibited potent lytic activity in a standard chromium release assay *in vitro*, indicating that they were functional (data not shown). Notwithstanding this fact, the apposition of cytolytic T cells with potential target cells did not result in induction of cellular damage. Therefore, functional "tolerance" had been induced *in vivo* but not *in vitro*, at least with regard to lytic activity. To determine whether the inclusion of inflammatory signals would alter the outcome of the response, mice were infected with VSV-OVA at the time of OT-I transfer. Four days after OT-I transfer and concomitant VSV-OVA infection, donor cells had expanded to a much greater extent in the MLN of IFABP-tOVA mice, as compared with their expansion in normal mice. This result showed that endogenous OVA served to potentiate the CD8 T-cell response to the VSV-OVA infection. The virus infection, along with OT-I transfer to the transgenic mice, also resulted in a transient weight loss in 232-6 mice and a sustained weight loss leading to death in most cases in 232-4 mice (36). In the absence of VSV-OVA infection, OT-I transfer did not cause weight loss (Fig. 4), and virus infection alone also had no effect on the weight of the mice or on the mucosal tissue.

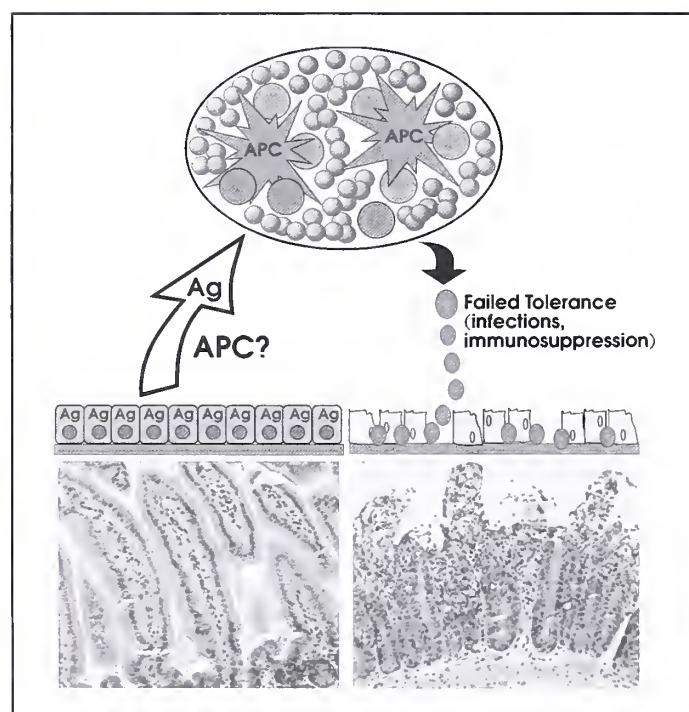


Fig. 4. Proposed pathway of acquisition of intestine-specific antigen and induction of immune tolerance or autoreactivity. Antigen is acquired by secondary lymphoid antigen-presenting cells from ova-expressing epithelium of 232 transgenic mice either via absorption from the gut lumen as material from intestinal epithelial cell turnover or from dying epithelial cells and migrate to the Peyer's patches and mesenteric lymph nodes. In the secondary lymphoid tissue, in the absence of inflammation, tolerance induction will occur primarily through deletion for CD8 T cells. When inflammatory signals are present, as in a virus infection, or dysfunction of regulatory cells, then the response is converted to a productive one that is potentially pathogenic. The photograph on the right shows a hematoxylin-eosin stain of a section from the ileum of a 232-4 mouse 4 days after OT-I cell transfer and concomitant virus infection.

Histologic examination of intestinal tissue after OT-I transfer and VSV-OVA infection revealed significant damage to the intestinal epithelium (Fig. 4). In 232-6 mice, destruction of duodenal and jejunal tissue was substantial with loss of epithelial cells, shortening of villi, and elongation of crypts (36). Crypt epithelial cells were not damaged, in keeping with the known expression pattern of IFABP. There was much less damage to ileal tissue and no effect on large intestinal tissue. In 232-4 mice, the disease was much more severe compared with that seen in 232-6 mice (Fig. 4). There was extensive destruction of epithelium in duodenal, jejunal, and ileal sections, although the latter sections were once again the least affected. The majority of these mice died of the disease within 6 days after infection. In those 232-4 mice that survived and in all 232-6 mice, intestinal tissue was histologically normal approximately 2 weeks after the destructive episode. These results clearly demonstrated that CD8 T cells are capable of antigen-specific recognition of IEC and of inducing IEC death.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The model described here will be invaluable for analysis of the stages of epithelial cell damage and repair inherent to the intestinal mucosal immune system. The mediators of cell death produced by CD8 T cells that act on IEC can be evaluated with the use of gene knockout mice and blocking antibodies. Moreover, the mechanisms by which tolerance versus autoimmunity are induced are readily testable in this well-defined and tissue-specific system. Future studies will focus on the interaction of CD4 T cells with IEC-expressed antigens and will provide further clues to the workings of mucosal tolerance and immunity. This model also presents a unique system in which factors influencing mucosal repair can be studied and that could have a direct impact on the repair of the epithelial damage inherent to cancer therapy. Since IEC damage can be induced selectively in enterocytes and regulated by antigen levels and, perhaps, by other factors such as T-cell number and virus dose, the system can be easily manipulated to examine the factors, immune or otherwise, of repair of mucosal tissue. By using other mucosa-specific promoters with distinct expression patterns, a further level of control can be attained. In sum, the study of immune-mediated mucosal injury and repair can be investigated in significant detail by using *in vivo* model systems that allow control of antigen expression and the immune response directed toward that antigen.

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NOTES

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Inflammatory Cytokines and Mucosal Injury

David A. Williams

The cause of mucosal injury in inflammatory bowel disease (IBD) is not clear but likely involves infectious agents or other toxins followed by an abnormal immune response in genetically susceptible individuals. The inflammatory cytokines appear to play a key role in both the susceptibility of some individuals and the tissue damage that accompanies IBD. The generation of transgenic and gene-targeted (knock-out) animals has provided invaluable information regarding the cytokines and cellular immune effectors that are important in IBD. Information from these and other preclinical animal models, such as those involving interleukin 11, has led to human trials testing novel therapies for IBD and other diseases in which inflammation of the gut mucosa is an important component. Thus, expression of inflammatory cytokines appears to be an important target for the development of novel therapies for IBD and other diseases in which intestinal mucosal damage occurs, such as mucositis and graft-versus-host disease. [J Natl Cancer Inst Monogr 2001;29:26–30]

The etiology of inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, remains unknown and is likely multifactorial. General theories of contributing factors include persistent or inciting infectious agents, a defective mucosal barrier, and abnormal host immune responses to infection or environmental antigens (1). Genetic epidemiologic studies and more recent genome mapping studies (2,3) indicate that inherited factors may contribute to individual susceptibility to IBD. While little direct evidence exists supporting the role of abnormal host immune response in IBD in humans, a large body of data in experimental animal models and phenotypes of animals deficient in specific genes generated by homologous recombination methods suggests that the immune system plays a key role in either initiating or maintaining the disease state (4,5). These observations have led to clinical studies targeting modification of specific immune regulators (Table 1). In the case of interleukin (IL)-11, a pleiotropic cytokine of mesenchymal origin, observations in animal studies of mucosal damage by chemotherapy agents led to subsequent human trials in IBD (6).

ABNORMAL HOST IMMUNE RESPONSES IN MUCOSAL DISEASE

A variety of studies in animals have led to the hypothesis that an abnormal host immune response is an essential feature of mucosal disease [reviewed in (7)]. These studies suggest that various initiating events in a genetically susceptible individual lead to an imbalance between proinflammatory and anti-inflammatory cytokines (Table 2). T-helper (CD4) cells under the stimulation of specific cytokines differentiate into two subsets of cells called T_{H1} or T_{H2} [reviewed in (8)]. In many studies, T cells of the T_{H1} subset appear to be an important mediator of mucosal inflammation. T_{H1} cells are induced by IL-12 and interferon gamma (IFN γ), whereas T_{H2} cells are induced by IL-

10 and IL-4. T_{H1} cells mediate various cellular immune responses, including macrophage activation, leading to the production of proinflammatory cytokines, including IFN γ , IL-2, IL-12, tumor necrosis factor (TNF), nitrous oxide (NO), and IL-1. T_{H2} cells mediate hypersensitivity responses, reduce macrophage activation, and stimulate antibody responses. The resulting effects of stimulation by T lymphocytes on a variety of other inflammatory cells (mast cells, neutrophils, and natural killer cells) lead to the production of a large number of soluble inflammatory mediators that are increased in IBD, including arachidonic acid metabolites, toxic phagocytic products (oxygen metabolites, nitric oxide, collagenases, etc.), toxic lymphocyte products, neuropeptides, and various components of the plasma proteolytic cascades (1,4,5,7,9).

The generation of various gene knockout and transgenic mouse strains has contributed substantial new understanding to the role of T cells in the development of IBD [Table 3 and reviewed in (9)]. In particular, IBD develops in mice with alterations in T-cell subpopulations and T-cell selection, including T-cell receptor-deficient (10,11), major histocompatibility complex class II-deficient (11), and severe combined immunodeficient mice restored with CD45RB^{HI} helper T cells (12,13); human leukocyte antigen (HLA)-B27 rats (14); mice with targeted disruption of cytokine genes, including IL-10 (15), IL-2 (16,17), and transforming growth factor- β (18,19); and mice lacking signaling proteins important in T cells, including α_2 subunit of G protein (20) or SMAD3 (21). Although these animals have a variety of specific defects in immune function, as noted by Powrie (4), all share a common feature in that, in each, the T-cell-dependent regulatory system that normally protects the gut is disrupted. One implication of these studies is that T lymphocytes play a critical role in the development and maintenance of oral tolerance (5). While direct evidence of the role of these genes in the development of IBD in humans is lacking, there are immunoregulatory features in Crohn's disease and ulcerative colitis, including a decreased ratio of IL-1ra to IL-1 in mucosal biopsy specimens from ulcerative colitis and Crohn's patients (22). IL-1ra is a circulating IL-1 receptor, which, if present at high levels, negatively regulates the effects of IL-1. In addition, some experimental evidence (23) suggests that monocytes from IBD patients may produce less IL-4, a key anti-inflammatory cytokine.

IBD IN IL-10-DEFICIENT MICE

Mice deficient in IL-10 have been especially useful in understanding the role of inflammatory cytokines and other factors in

Affiliations of author: Howard Hughes Medical Institute, Indiana University School of Medicine, Section of Pediatric Hematology/Oncology, Herman B Wells Center for Pediatric Research, and Department of Pediatrics, Indiana University School of Medicine, Indianapolis.

Correspondence to: David A. Williams, M.D., Herman B Wells Center for Pediatric Research, 1044 West Walnut St., Rm. 402, Indianapolis, IN 46202 (e-mail: dwilliam@iupui.edu).

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Table 1. Clinical trials for inflammatory bowel disease involving cytokines

- Anti-interleukin 12
- Interleukin 10
- Interleukin 11
- Antitumor necrosis factor- α

Table 2. Inflammatory mediators postulated to be involved in mucosal pathology*

- Pro-inflammatory
IL-1, IL-6, TNF- α , IFN- γ , IL-2
- Anti-inflammatory
IL-1ra, TGF- β , IL-4, IL-10, IL-11

*IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; IL-1ra = circulating IL-1 receptor; TGF = transforming growth factor.

Table 3. Transgenic animals with inflammatory bowel disease*

- Alterations in T-cell subpopulations or selection
TCR (α/β)
MHC class II
SCID mouse restored with CD45RB^{HI} helper T cells
HLA-B27 rat
- Cytokine knockout mice
IL-2
IL-10
TGF- β
- Signaling proteins
G protein subunit (G α 2)
SMAD3

*TCR = T-cell receptor; MHC = major histocompatibility complex; SCID = severe combined immunodeficient; HLA = human leukocyte antigen; IL = interleukin; TGF = transforming growth factor.

the development of mucosal inflammation. IL-10 was initially identified as an activity produced by T_{H2} cells that inhibited the production of cytokines by T_{H1} cells (24). However, IL-10 has effects on a broad range of immune functions and is a potent suppressor of macrophage activation, inhibiting production of inflammatory cytokines, such as IL-1, IL-6, and TNF- α . Kuhn et al. (15) described the development of mice deficient in IL-10 generated by homologous recombination targeting the IL-10 gene in embryonic stem cells. Surprisingly, these mice developed normal numbers of B and T lymphocytes and demonstrated a normal immune response to T-cell-dependent immunizations. However, these mice developed an abnormal T_{H1} response to nematode infection with increased production of INF- γ and IL-5. The animals were also growth retarded and developed a microcytic anemia that was likely caused by iron deficiency. The principal histopathologic finding in anemic and low-weight mice was a chronic enterocolitis involving the entire intestinal tract. The pathologic lesions in the intestine included mucosal inflammation, degeneration of the intestinal mucosa, and marked thickening of the mucosal wall associated with excessive regenerative hyperplasia. Mucosal surfaces demonstrated desquamation of the apical epithelia with superficial erosions and inflammatory exudates. Extensive histiocytic, lymphoid, macrophage, neutrophil, eosinophil, and plasma cell infiltration was seen in the lamina propria and submucosa regions. The duodenum showed

the most extensive abnormalities, but changes were seen throughout the gastrointestinal tract. These pathologic changes were attenuated in mice bred in specific-pathogen-free (SPF) conditions, suggesting a role for microbial antigens in the severity or progression of the IBD.

In a subsequent study, Berg et al. (25) demonstrated disease progression that occurred in mice kept in SPF conditions. These studies documented the fact that inflammatory changes first occurred in the cecum and ascending and transverse colon and ultimately involved the rectum and small intestine. Prolonged disease was associated with transmural lesions and increased incidence of adenocarcinomas. Mechanistically, Berg et al. (25) demonstrated increased amounts of inflammatory cytokines (IL-1 α , TNF- α , IL-6, IFN γ , and NO) produced in colonic cultures of IL-10-deficient mice. Purified CD4⁺ T cells derived from the colons of these mice also produced substantially more IFN γ . Treatment of mice with anti-IFN γ antibodies prevented colitis from developing, and administration of IL-10 substantially reduced colitis, duodenitis, and the incidence of colorectal adenocarcinoma. These studies also demonstrated genetic differences in disease susceptibility, since a marked difference in the development of intestinal lesions was seen in multiple inbred mouse strains. Kullberg et al. (26) more recently demonstrated that IL-10-deficient mice reared in SPF conditions that were experimentally infected with *Helicobacter hepaticus* developed chronic colitis associated with a T_{H1} cytokine response (TNF- α , IFN γ , and NO). In this experimental infection with one specific microbe, neutralization of IFN γ or IL-12 *in vivo* with antibodies resulted in a substantial reduction in intestinal inflammation. In summary, the data generated from IL-10-deficient mice suggest a key role for proinflammatory cytokines, inheritable factors, and microbial antigens in the development and progression of IBD.

IL-11 IN THE TREATMENT OF INFLAMMATORY DAMAGE OF THE INTESTINE

As noted above, IL-11 is a pleiotropic cytokine of mesenchymal origin. The cloning of the complementary DNA (cDNA) responsible for IL-11 activity followed the development of more than 60 immortalized stromal cell lines from primate bone marrow by using a recombinant retrovirus vector expressing simian virus 40 large T antigen (27). Of these cell lines, one designated PU-34 demonstrated the capacity to generate megakaryocytic cells from human CD34⁺ cells when used in long-term cultures. Conditioned medium from this cell line was shown to support proliferation of an IL-6-dependent plasmacytoma cell line, T1165, in the presence of excess neutralizing antibodies to IL-6. Because of data suggesting a role for IL-6 in megakaryocyte colony growth in some systems, this activity was further studied. With the use of expression cloning methods and the IL-6-dependent plasmacytoma cell line, an IL-11 cDNA was cloned (along with multiple IL-6 cDNA clones) and was shown to have megakaryocyte colony-forming activity *in vitro* (28). Subsequently, IL-11 was shown to stimulate the recovery of platelets *in vivo* after cytoablative therapy (29) and in normal mice (30). Effects on platelet production were seen in a variety of preclinical models and in early-phase trials in humans [for a review, see (31)]. In 1998, the U.S. Food and Drug Administration approved IL-11 (Neumega) as the first pharmacologic agent for the treatment of chemotherapy-induced thrombocytopenia.

Human IL-11 is a 199-amino-acid protein with a molecular weight of approximately 19 kd [reviewed in (32)]. The gene

maps to 19q13.3-q13.4 in a region that contains several zinc finger genes and spans 7 kilobases (33). The protein, unlike many cytokines, is not glycosylated and has no cysteine residues or potential N-linked glycosylation sites. The cytokine probably has a structure with a four-helix bundle topology with two receptor-binding sites located in the carboxyl terminus (34). Many mesenchymal cell lines have been shown to express IL-11, whereas IL-11 messenger RNA is abundant in the murine testis, hippocampal neuronal cells, and motor neurons and sympathetic neurons of the spinal cord (35). IL-11 is a member of the IL-6 cytokine superfamily. The receptor for IL-11 contains a common GP130-signaling subunit and a specific α chain (36). Binding of IL-11 stimulates receptor dimerization, activation, and phosphorylation of Jak/Stat proteins.

In an effort to develop more severe thrombocytopenia in a mouse model in which to test the stimulatory activity of IL-11, Du et al. (6) treated mice with the combination of total-body irradiation and 5-fluorouracil (5-FU). Mice treated with this combination therapy and IL-11 were noted to survive at significantly ($P = .01$) higher frequency than control mice. Neither an increase in leukocyte counts nor significant changes in bleeding explained the survival differences. Because deaths occurred rapidly after cytoablative therapy, examination of the gut was undertaken; this analysis demonstrated marked changes in the mucosal architecture. Control mice demonstrated significant ($P = .01$) shortening of villus length and areas of ulcerations accompanied by enteric bacterial foci in the liver. IL-11 treatment was associated with increased villus length, preserved villus/crypt ratios, and reduced incidence of hepatic bacterial foci. Subsequent studies by many laboratories have demonstrated similar results in multiple models of gut injury, including ischemia (37), burn (38), short gut (39), trinitrobenzene sulfonic acid (40), HLA-B27 rat (41), 5-FU-induced mucositis (42), radiation therapy alone (43), and graft-versus-host disease (GVHD) (44). One study suggests a direct effect on mucosal cells *in vivo* (45).

Studies on GVHD provide evidence of the mechanisms by which IL-11 may be acting in these varied models. GVHD is a multisystem disease in which donor T cells directed against host antigen(s) mediate inflammation and tissue damage. Common end organs affected in GVHD include skin, gastrointestinal tract, liver, and blood cells (46). Clinical symptoms include exfoliative skin rashes, hepatitis, diarrhea, weight loss, immune-mediated blood cell destruction, and increased incidence of opportunistic infections. Although the specific host antigens that are mediators of GVHD have not been identified, modulation of the incidence and severity of the disease occurs with T-cell depletion of the donor stem cell preparation (46). Hill et al. (44) demonstrated that administration of IL-11 in an animal model of GVHD significantly ($P = .05$) reduces the incidence and severity of intestinal complications and leads to increased survival of treated mice. These changes in the intestinal mucosa were associated with a substantial reduction in IFN γ and IL-2 secretion and an increase in IL-4 secretion. The T_{H2} polarization of the T-cell response also led to decreased IL-12 production in mixed lymphocyte cultures *in vitro* (44). It is interesting that systemic levels of TNF- α , a potent inflammatory cytokine, were significantly ($P = .01$) reduced by IL-11 treatment. The authors hypothesized that IL-11 treatment reduced GVHD morbidity and mortality by polarization of donor T cells (to T_{H2} response), protection of the small intestine, and suppression of inflammatory cytokines.

Additional studies have also provided evidence that IL-11 has potent anti-inflammatory effects and may be acting in the gut by modulating macrophage cytokine production. Trepicchio et al. (47) have demonstrated that administration of IL-11 to lipopolysaccharide-treated mice reduced TNF- α , IL-1, and IFN γ levels *in vivo*. Treatment of isolated macrophages *in vitro* with IL-11 led to reduced production of these same cytokines and IL-12 p40 and NO. Subsequent studies demonstrated a dose-dependent decrease in IL-12 production by macrophages after combined IFN γ /Staphylococcus aureus stimulation *in vivo*. This decrease in expression of IL-12 was caused by transcriptional regulation (48). This transcriptional effect could be due to increased expression of I κ B- β and I κ B- α with subsequent decreased nuclear translocation of NF- κ B in macrophages (49). Thus, studies support the hypothesis that IL-11 may be beneficial in IBD by a combination of direct effects on enterocyte production/survival and modulation of immune responses, including systemic reduction in inflammatory cytokines.

Early studies in humans have supported the preclinical data presented above. A multicenter, double-masked, placebo-controlled study in 76 patients with IBD demonstrated the expected increase in platelet counts and showed that 42% of IL-11-treated patients had a positive clinical response in terms of IBD symptoms, versus 7% in the placebo group (50). This dose-escalation study showed minimal toxic effects with IL-11 given subcutaneously two times per week. The response was seen at a dose of 16 μ g/kg per week. A multicenter, double-masked, placebo-controlled phase II study has been recently reported in abstract form (51). In this study, 148 patients with active disease (Crohn's disease activity index [CDAI] >220) were given placebo versus IL-11 in two schedules (15 μ g/kg once a week or 7.5 μ g/kg twice a week). Results in the once-a-week group showed a trend toward decreased mean percentage CDAI (32% versus 18% in placebo) and a substantial increase in remission rate (37% versus 16% in placebo, $P < .05$). Treatment using the twice-a-week schedule was effective but was associated with increased rates of side effects, including headache, edema, and increased platelet counts. These side effects were not noted at any increased frequency in the once-a-week treatment group compared with the placebo group. Thus, IL-11 appears to be both safe and effective in inducing remissions in a subset of patients with active Crohn's disease. On the basis of these early clinical studies, a multi-institutional phase III trial is now under way (Schwertschlag U; personal communication).

The expression of inflammatory cytokines appears to play a critical role in the development and progression of IBD. On the basis of a number of preclinical animal models, in which the expression of these cytokines is modulated, and early human clinical trials, expression of inflammatory cytokines appears to be an important target for the development of novel therapies for IBD and other diseases in which intestinal mucosal damage occurs, such as mucositis and GVHD.

RELATIONSHIP OF INFLAMMATORY CYTOKINES AND MUCOSAL INJURY IN CANCER

The generation of mucositis and bowel injury accompanying chemotherapy and radiation therapy used in cancer treatment continues to be a major source of morbidity for many patients. In addition, these side effects of cancer therapy can frequently have adverse consequences on dose intensity and, therefore, compromise multimodality approaches to the cure of cancer. Mucosal

injury is also a striking component of GVHD seen in the post-allogeneic stem cell transplant setting, a process that substantially increases morbidity and mortality in those using this therapeutic approach for the treatment of cancer. Although direct cytotoxicity of many chemotherapy agents and radiation to intestinal mucosal cells undoubtedly plays a major role in the development of mucositis and other intestinal complications of these therapies, inflammatory cytokines, as discussed in this article, probably also contribute substantially to the severity and maintenance of injury.

FUTURE RESEARCH DIRECTIONS

The use of transgenic and gene-targeted mice will continue to elucidate important mechanisms of mucosal injury. These studies, therefore, provide logical and relevant targets for future pharmacologic intervention. Future studies are needed to continue to define the basic mechanisms of mucosal cell growth and differentiation but also should aim to translate these findings into patient-focused research in the treatment of IBD, mucositis, and other gastrointestinal complications of cancer therapies

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NOTES

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Infection and Mucosal Injury in Cancer Treatment

Shahab A. Khan, John R. Wingard

The oral and gastrointestinal mucosa acts as an important mechanical barrier that prevents local or systemic invasion by microorganisms. Cytotoxic chemotherapy-induced mucosal injury (MI) of oral cavity and intestinal epithelium occurs in many patients treated for malignancy. Compromise of the mucosal barrier can contribute to local invasion by colonizing microorganisms and, subsequently, to systemic infection. Historically, gram-negative bacteremia has been the most problematic bacterial infection in neutropenic patients, but its incidence has reduced over time because of the use of prophylactic antibiotics. There has been a shift in the type of infecting organisms responsible for bacteremia in these patients, from predominantly gram-negative organisms to gram-positive cocci. The viridans group of streptococci is composed of the most frequent bacterial pathogens associated with MI. When speciated, oral colonizers such as *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis* II are the most frequently identified pathogens. Other systemic infections caused by vancomycin-resistant enterococci, *Stenotrophomonas maltophilia*, and *Candida* species have also been associated with MI after cancer treatment. Infection can also exacerbate MI after cancer treatment. The best recognized example is herpes simplex virus type 1 (HSV-1). Latent virus is frequently reactivated in HSV-seropositive patients; this reactivation leads to stomatitis, which can be indistinguishable from MI caused by cytoreductive therapies. Antiviral prophylaxis or treatment can control the virus-induced MI and bring about overall amelioration of MI. Recognition of this infectious cause of MI is important in order for clinicians to anticipate and minimize oral toxicity and to facilitate optimal delivery of the antineoplastic regimen. [J Natl Cancer Inst Monogr 2001;29:31-6]

Mucosal injury (MI) can lead to a variety of systemic consequences. These include impaired oral intake of fluid and nutrients, leading to dehydration and malnutrition; pain; nausea; vomiting; abdominal cramping; and diarrhea. The mucosa of the oral cavity and gastrointestinal (GI) tract also serves as an important mechanical barrier that helps to prevent a local or systemic invasion of various microbes and the absorption of microbial products that are normally present in the oral cavity and the lumen of the gut (1). Derangement in the barrier function of the GI tract plays a central role in the pathophysiology of systemic infection, shock, and sepsis syndrome. In this article, we will examine two propositions. The first is that MI is a major contributor to the development of systemic infection by commensal colonizing organisms and that this presents a serious challenge to optimal management of the cancer patient. The second proposition examines the notion that certain microorganisms exacerbate MI, which, in turn, can increase the susceptibility for systemic infection from other commensal organisms. Infectious causes of MI are indistinguishable from cytotoxic drug-induced MI and can be confused with MI from the anti-

neoplastic regimen. Infection-induced MI may necessitate dose reduction or modification of the antineoplastic regimen, which may compromise the ultimate benefits of the treatment regimen.

EFFECTS OF CANCER TREATMENTS ON THE ORAL AND GI MUCOSA

Cytotoxic chemotherapy is known to cause MI both in the oral cavity (2-6) and to mitotically active intestinal crypt cells (7). The manifestations of oral mucositis include erythema, ulcer formation, bleeding, and exudates. Methotrexate (7), 5-fluorouracil, cisplatin (8), cytarabine (9), etoposide, and radiation therapy (XRT) (10) have been shown to have mucosal-damaging effects. Most of the patients treated for head and neck cancer and almost half of the patients receiving chemotherapy for non-head and neck cancer develop oral complications (11).

The course of oral mucositis after standard- or high-dose chemotherapy parallels the neutropenia that occurs following such therapy. The onset of oral mucositis occurs near the nadir of neutrophil count, and its resolution parallels hematologic recovery (12).

Slavin et al. (9) described the natural history of cytotoxic therapy-induced intestinal damage. Initial injury began during the first week of cytotoxic therapy and was characterized by replacement of normal crypts of mucous-secreting cells by atypical undifferentiated cells. During subsequent weeks, the injury progressed to a second stage, which consisted of cellular necrosis, a lack of mitotic activity, disappearance of villous surface, and complications by various infections. Finally, the recovery phase followed, when mitotic activity returned and cells regenerated, differentiated, and covered the denuded surface. There are several studies (13) of D-xylose absorption tests that have been used as a measure of functional integrity of the intestinal mucosal barrier. Studies in patients with acute myeloid leukemia (AML) receiving remission induction therapy have shown malabsorption of D-xylose during weeks 2 and 3 after chemotherapy, secondary to gut epithelial damage (14,15). The magnitude of intestinal epithelial damage as measured by D-xylose malabsorption was strongly correlated with the induction regimen.

The effect of radiation therapy on oral cavity primarily results from local tissue changes. These changes are initiated by a reduction in the proliferation of basal epithelial cells, causing atrophy (11). The damage of connective tissue may lead to an increase in vascular permeability and tissue edema (10).

Oral complications of cancer chemotherapy may be direct somatotoxicity of chemotherapy against basal epithelium; indi-

Affiliation of authors: University of Florida College of Medicine, Gainesville.

Correspondence to: John R. Wingard, M.D., Division of Hematology, University of Florida College of Medicine, 1600 SW Archer Rd., P.O. Box 100277, Gainesville, FL 32610-0277 (e-mail: wingajr@medicine.ufl.edu).

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rect somatotoxicity through the patient's inability to contain local, minor oral disease during myelosuppression; or a combination of both (11). Local infection can produce inflammatory changes that further exacerbate MI.

The degree of MI is dependent on the dose intensity of the treatment regimen. Mucositis is particularly frequent in the bone marrow transplant (BMT) population because of the intensive conditioning regimen. Approximately 75% of patients develop some degree of mucositis after the conditioning regimen, which consists of high-dose chemotherapy or combined chemoradiation. Over two thirds of patients with leukemia and one third of those with non-Hodgkin's lymphoma develop MI (11). Patients with solid tumors are at lower risk of developing MI (40%), except for patients with head and neck cancer who receive combined XRT and chemotherapy. Virtually all of these patients develop mucositis. The effects of XRT on the mouth primarily result from local changes. Consequently, the total dose of XRT to the oral cavity and dose rate are directly related to the extent of MI. The MI is noted at a level of 20 Gy when XRT is administered at a rate of 200 cGy daily (11).

MI of the oral cavity is frequently accompanied by oral infections. Viral, bacterial, and fungal infections are all common. In some clinical settings, systemic bacterial or fungal infections may be more common in patients with mucositis.

MI AS CONTRIBUTORY FACTOR TO SYSTEMIC INFECTION

Bacterial Infections

Bacteremia from gram-negative rods has been the most problematic bacterial infection in chemotherapy-induced neutropenia. The GI tract is a major source of bacteria in patients who develop MI as a result of chemotherapy (16). Between 25% and 50% of cases of septicemia in neutropenic cancer patients appear to originate from oral colonizing bacteria (17).

The incidence of gram-negative bacterial infections in neutropenic patients has decreased over time, perhaps because of both the prophylactic use of broad-spectrum antibiotics in neutropenic patients and the empiric use of systemic broad-spectrum antibiotics at first sign of fever in neutropenic patients (18) (Table 1). Nevertheless, studies (18–25) have shown that bacteremia caused by gram-positive organisms is becoming more common. At present, gram-positive bacteria represent the overwhelming majority of neutropenic systemic infections. Furthermore, substantial proportions of these gram-positive bacterial pathogens are viridans group streptococci.

Viridans streptococci are now the second most common ge-

nus of bacteria isolated from blood culture after coagulase-negative staphylococci. They can be responsible for up to 39% of bacteremia cases in neutropenic population (26). Several authors (27–29) have suggested that oropharyngeal lesions were the most probable portal of entry for viridans streptococci that caused bacteremia. Other investigators (30) have suggested that the rest of the digestive tract, particularly the stomach and lower respiratory tract, might also be portals of entry. In a recent review of literature on bacteremia caused by viridans streptococci in neutropenic patients (26), the most frequently isolated species in blood culture were *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguis* II. Various risk factors have been identified, and the presence of oropharyngeal mucositis was a statistically significant independent factor in most of these studies (26,32,35). Other risk factors included severe neutropenia, prophylactic antibiotic treatment with co-trimoxazole or quinolone, chemotherapy involving high doses of cytarabine, GI toxicity requiring antacids or H₂ blockers, and heavy colonization by viridans streptococci (27–34). Bochud et al. (32) reviewed 26 episodes of viridans streptococcal bacteremia that occurred in 25 neutropenic patients undergoing intensive chemotherapy for hematologic malignancies. Multivariate analysis of predisposing factors showed that the presence of mucositis was an important independent risk factor for the development of viridans streptococcal bacteremia. Pharyngeal lesions were statistically significantly more frequent in case patients (85%) than in the control patients (55%) ($P = .01$). Multivariate analysis of risk factors showed that mucositis was among the three independent predictors for the development of viridans streptococcal bacteremia ($P = .02$).

Ruescher et al. (35) reported on 24 patients who were treated with high-dose chemotherapy and an autologous BMT for hematologic malignancies and who had developed bacteremia with α -hemolytic streptococci. Of these 24 patients with bacteremia, 14 (62%) had ulcerative mucositis, compared with 16 (36%) of 45 patients in the control population ($P < .05$). Patients with ulcerative mucositis were found to be three times as likely to develop α -hemolytic streptococcal bacteremia as those without ulcerative mucositis (odds ratio = 3.02).

Streptococcal organisms are the most frequent bacterial pathogens associated with MI. However, systemic infections by other bacteria and fungi have also been implicated as sequelae of MI.

Vancomycin-resistant enterococci (VRE) are rapidly increasing causes of infection in hospitalized patients and are associated with considerable morbidity (36). Mucositis has been implicated as a possible contributory factor associated with invasive VRE infection. In one study reported by Kuehnert et al. (37), 738 cancer patients admitted into the hospital had at least one stool specimen obtained for VRE. Nineteen cases of VRE bacteremia were identified. When case patients were compared with control patients, the presence of mucositis, among other factors, was statistically significantly associated with VRE bloodstream infection ($P < .01$) in univariate analysis. When the independent importance of various risk factors identified in univariate analyses was tested in multivariate analysis using logistic regression models, only mucositis remained statistically significantly associated with VRE bacteremia. Furthermore, when the severity of mucositis was assessed quantitatively, the risk of VRE bacteremia increased with increasingly severe mucositis ($P < .003$); this finding remained valid after adjusting for severity

Table 1. Infectious pathogens encountered in cytotoxic-induced myelosuppression†

Systemic pathogen arising from gut*	Relative frequency	Relative severity
GNR	+	+++
GPC	+++	++
<i>Candida</i>	++	+++
<i>Aspergillus</i>	++	+++
HSV	+++	+
CMV	+	+++

*GNR = gram-negative rods; GPC = gram-positive cocci; HSV = herpes simplex virus; CMV = cytomegalovirus.

†+, less frequent; ++, frequent; +++, more frequent.

of illness and degree of neutropenia. Kuehnert et al. hypothesized that the association of mucositis with VRE bacteremia may be due to diffuse GI mucosal breakdown, which promotes bloodstream entry by gut-colonizing VRE.

In recent years, the emergence of increasing bloodstream anaerobic infections in neutropenic patients, formerly rare, has been described. Most of these patients have oral mucositis or periodontal disease (38).

Labarca et al. (39) reported *Stenotrophomonas maltophilia* bacteremia in a cluster of eight allogeneic BMT patients. In addition to other associated factors identified when infected patients were compared with control patients, severe mucositis was identified as one of the risk factors ($P = .028$).

Fungal Infection

Invasive fungal infections are frequent in patients undergoing cancer chemotherapy that results in prolonged neutropenia and after a BMT (40). As many as 40% of patients undergoing BMT develop invasive fungal infection when neutropenia persists for more than 20 days (41).

Candida and *Aspergillus* sp. are the most frequent causes of fungal infection in leukemia patients undergoing chemotherapy and in BMT patients. Invasive fungal disease in these patients is associated with a high mortality rate (approximately 50%–90%) (42,43). *Candida* species are commensal organisms that reside normally on the oral mucosa and in the lumen of the GI tract. They not only can cause local infection of the oral mucosa, which is painful, but also can result in esophageal candidiasis or in systemic dissemination. Systemic fungal infections are difficult to recognize and respond poorly to treatment (44). The intact mucosa is an important host defense against systemic *Candida* infection in neutropenic patients (14).

Wingard et al. (44) reported on 89 consecutive patients treated intensively for leukemia or undergoing BMT for a 12-month period. They observed 18 episodes of *Candida* sepsis in 17 patients (19%). Three (5%) of 60 patients colonized by *Candida albicans* in their mucosa became infected, while 14 (56%) of 25 patients colonized by *C. tropicalis* became infected ($P \leq .001$). These data suggest that *C. tropicalis* is a more virulent systemic pathogen than *C. albicans* in neutropenic cancer patients, despite being a less frequent colonizer of mucosal surface (45). When examined in animal models of *Candida* virulence, no difference in virulence was noted between *C. albicans* and *C. tropicalis* when the organisms were given intravenously. However, after the organisms were inoculated into the esophagus in mice given chemotherapy that induced damage of the gut mucosa (Fig. 1) and neutropenia, *C. tropicalis* isolates were substantially more virulent than *C. albicans* isolates. In dose-response assays, systemic invasive infection occurred at inoculation doses more than 100-fold less with *C. tropicalis* isolates than with *C. albicans* isolates (46,47).

Bow et al. (14) studied the relationship of cytotoxic regimens with intestinal mucosal damage and fungal colonization in the pathogenesis of invasive fungal disease in 138 patients undergoing induction therapy for untreated AML. They used weekly D-xylose absorption tests (13) for evaluation of the functional integrity of the upper GI tract and to measure small intestinal epithelial damage. Their results suggested that pathogenesis of invasive fungal disease is linked to cytotoxic therapy-related gut epithelial damage in the setting of fungal colonization of the gut. Patients in whom invasive fungal disease developed had

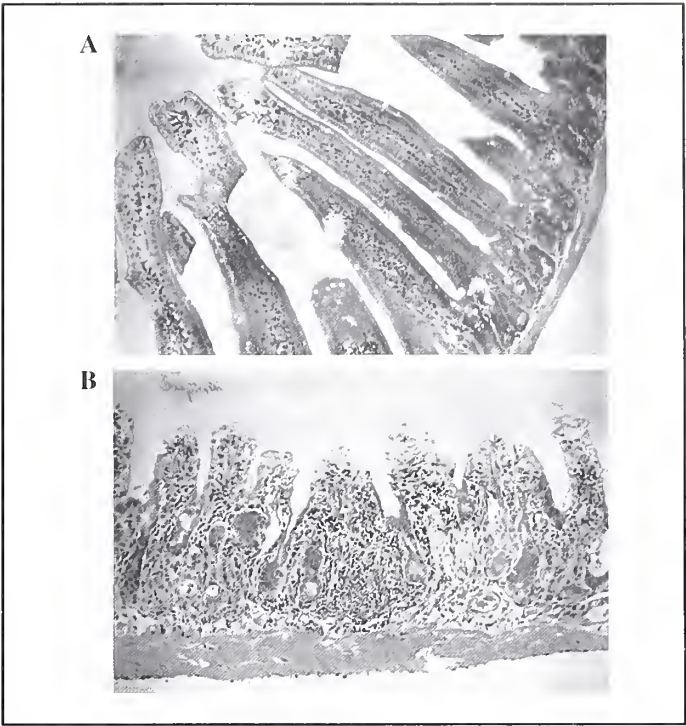


Fig. 1. A) Cross-sectional histology of normal gastrointestinal tract. Note the elongated villi, single-cell epithelial lining, lack of cellular infiltrate in the submucosa, and scant submucosal blood vessels. B) Following cytarabine, the villi are markedly blunted, and there is denudation of the epithelial mucosa, marked inflammatory infiltration, and dilatation of submucosal blood vessels, with scattered hemorrhages.

lower serum D-xylose levels (indicative of greater intestinal epithelial damage), with the maximal difference noted at weeks 2 ($P = .0288$) and 3 ($P = .0019$) of chemotherapy, than did uninfected patients. Bow et al. speculated that damaged mucosal surface may facilitate infection by promoting adherence, local proliferation, and translocation of microorganisms colonizing these surfaces. In this study, gut epithelial damage was maximal during weeks 2 and 3 of induction therapy, which was coincidental with the neutrophil nadir. In another report (13), neutropenic colitis and hepatosplenic fungal infection were also correlated with the D-xylose malabsorption. The mean serum D-xylose levels during week 2 of chemotherapy in AML patients were lower among subjects who developed neutropenic enterocolitis ($P = .002$) and hepatosplenic candidiasis ($P = .002$) (15). Neutropenic enterocolitis was strongly correlated with the development of candidemia ($P = .005$).

Various other reports have identified mucositis as a risk factor for fungemia among the patients receiving antineoplastic therapy. In one report (48) of 41 episodes of breakthrough fungemia occurring in cancer patients receiving antifungal prophylaxis, mucositis was identified as one of the risk factors for breakthrough fungemia (34.2% versus 13.1%; $P < .05$).

While each of these studies had methodological differences, each supports the concept that MI offers a portal of entry into the systemic circulation for commensal oral and GI bacteria. Thus, the ability to treat and prevent the severe MI would be an important tool to decrease the rate of bacterial infections in this patient population.

These studies in aggregate suggest that systemic infections resulting from MI occur in more intensively treated patients

(acute leukemia, induction therapy, and BMT) or are more prevalent in regimens that cause greater degrees of MI and that children are as vulnerable as adults. The reasons are not clear: Oral commensal organisms appear to be more frequent systemic pathogens than GI-colonizing organisms, despite the fact that there is a substantially greater burden of gut-colonizing organisms compared with oral colonizers and the fact that there is a much greater surface area of gut mucosa compared with oral mucosa.

INFECTION CONTRIBUTING TO MI

Herpes simplex virus type 1 (HSV-1) causes the most common symptomatic oral viral infection. HSV seropositivity is an indicator for latent or persistent infection, which may reactivate from a variety of stimuli such as chemotherapy or radiotherapy. This risk for reactivation correlates with the dose intensity of antineoplastic therapy. Reactivation occurs in up to 70%–80% of seropositive BMT and acute leukemic patients (49,50). Reactivation rates are lower in less intensively treated patient groups. HSV reactivation occurs in 38%–60% of non-Hodgkin's lymphoma patients under treatment and in 15%–20% of patients receiving chemotherapy or radiotherapy for head and neck cancer (51–62). The frequency of HSV reactivation in various antineoplastic treatment settings, especially solid tumor treatment regimens, is not well established. In many patients who are seropositive for HSV, the virus is reactivated after the chemotherapy (63). Resultant HSV-induced mucositis may be difficult to differentiate from MI from direct damage caused by chemotherapy, since the telltale labial blister, the pathogenomic feature of reactivation of HSV, may not be present. Deep and extensive oral ulcerations may occur because of HSV-1 (Fig. 2). In patients treated with high-dose chemotherapy with BMT or after intensive chemotherapy for leukemia, HSV-1 mucosal infection can also spread contiguously along the mucosal surface, resulting in esophagitis, tracheitis, or pneumonitis.

It is frequently difficult to distinguish between infectious and noninfectious oral mucositis caused by chemotherapy or irradiation. For example, when phase I dose escalation studies were performed for etoposide in a stem cell rescue setting, severe mucositis was reported to be the dose-limiting toxic effect (64). When phase I dose-escalation studies of etoposide were repeated with acyclovir prophylaxis to prevent HSV reactivation, the

maximally tolerated dose of etoposide, evaluated at 50% higher doses (65,66), was not achieved, clearly indicating that much of the formerly described MI attributed to etoposide-direct cytotoxicity was instead caused by reactivation of HSV. Ulcerative mucositis is still seen after administration of etoposide with acyclovir prophylaxis, especially at the high doses used in conditioning regimens in the BMT setting, but it is less severe.

Acyclovir, a nucleoside analogue, which is selectively phosphorylated by a virus-specified thymidine kinase targeting the viral DNA polymerase, is highly effective in preventing MI from HSV-I and has been shown to be effective prophylactically in prospective randomized trials (51,54,66,67). Before acyclovir's prophylactic use, HSV-infected patients had mortality rates from HSV-I infection as high as 5%–10% after BMT (68). Acyclovir prophylaxis can have secondary benefits in the reduction of the risk for systemic infection from streptococcal bacteria colonizing the mucosa. This was amply illustrated in 60 consecutive BMT patients in which the risk for streptococcal bacteremia was 25% in 30 patients not treated with acyclovir prophylaxis but 0% in 30 consecutive patients in which acyclovir prophylaxis was given (69).

Oral cytomegalovirus (CMV)-associated infection of the lip, labial mucosa, tongue, and pharynx has rarely been described in immunocompromised patients (70,71) and can be an infrequent infectious cause of MI. Some reports have described CMV infection of the tongue following BMT (72). CMV esophagitis and gastritis, while more common in acquired immune deficiency syndrome, are less frequently seen after BMT. Few case reports have described CMV esophagitis or gastritis either accompanying the more commonly seen CMV pneumonitis or coinfecting with HSV in patients receiving immunosuppressive therapy for treatment of graft-versus-host disease (73–75). CMV infection is an infrequent cause of colitis in BMT patients (76).

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

We conclude that the intact mucosa is an important host defense against systemic infection in neutropenic patients. MI is an important and identifiable risk factor for various bacterial and fungal infections, including viridans streptococcal, enterococcal, anaerobic bacteria, and certain gram-negative bacteria, in patients receiving cytotoxic chemoradiotherapy for the treatment of various malignancies. Moreover, the risk of invasive fungal disease is linked to cytotoxic therapy-related oral and gut epithelial damage in the setting of fungal colonization of the oral cavity and the gut.

The distinction between an infectious etiology of MI as opposed to regimen-related tissue damage is crucial to the optimal delivery of the antineoplastic regimen. Direct cytotoxicity during the course of repeated cycles of chemotherapy may necessitate dose reduction in subsequent courses of treatment. Such dose reductions may compromise the ultimate therapeutic control of the underlying neoplasm, since dose intensity has been shown in a number of studies (77–80) of certain neoplasms to affect not only remission rates but also survival. If an infectious etiology for MI were the case, then treatment of the infection during that given course would be appropriate, and subsequent secondary prophylaxis during subsequent courses of treatment to suppress further reactivation and MI would be appropriate to facilitate the delivery of the entire treatment dose. Moreover, amelioration of MI would make the antineoplastic regimen more

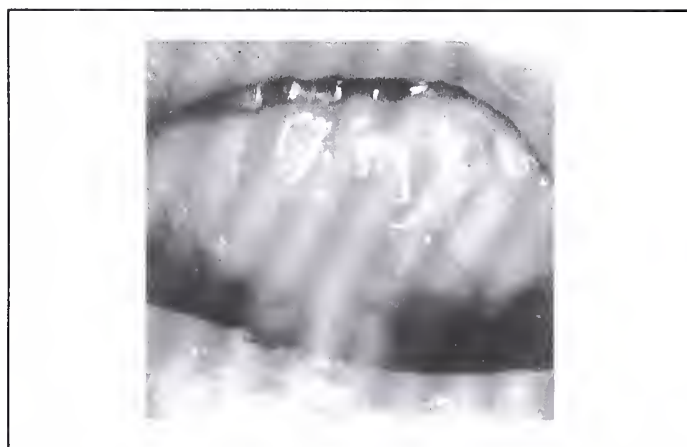


Fig. 2. Diffuse ulcerations of the oral mucosa after cytotoxic chemotherapy.

palatable to the patient and facilitate better compliance. At present, no specific therapies are proven to be effective to either treat or prevent MI secondary to cytotoxic chemoradiotherapy. Thus, new modalities to treat and prevent severe MI and the ability to understand the early steps in pathogenesis of infections at the mucosal barrier would improve the outcome of these groups of patients, reducing the treatment-related morbidity and mortality.

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NOTE

Editor's note: J. R. Wingard is a consultant for Intrabiotics and Amgen and is an investigator in a clinical trial by Intrabiotics to test a product that may be used in the prevention of mucositis.

Biology of Mucosal Pain

Christine Miaskowski

Pain is experienced when injury to mucosal tissues occurs. Although the neurobiology of mucosal pain has not been fully elucidated, research has demonstrated that the oral mucosa contains primary afferent nociceptors that respond to thermal, mechanical, and chemical stimuli. Inflammation occurs during the initial phase of mucosal injury caused by stomatotoxic chemotherapy or radiation therapy. This article reviews the mechanisms that underlie acute pain in inflamed cutaneous tissue and summarizes the major mediators that activate and sensitize primary afferent nociceptors. Recommendations for future research to elucidate the neurobiology of mucosal pain throughout the gastrointestinal tract are presented. [J Natl Cancer Inst Monogr 2001;29:37-40.]

Pain is a major clinical problem for most patients who sustain mucosal injury from cancer chemotherapy or radiation therapy. Little information is available, however, on the mechanisms of pain associated with mucosal injury in patients with cancer. Therefore, much of the information provided in this article on the mechanisms of pain associated with mucosal injury is extrapolated from research studies that have evaluated pain mechanisms in the presence of inflammation in cutaneous tissues.

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (1). Although it is recognized that pain associated with mucosal injury in patients with cancer includes an emotional component that is related to the cancer diagnosis and the stress of the treatment, this article focuses on the sensory portion of the pain experience and describes the biochemical changes that occur in response to tissue injury and that result in the sensation of pain.

TYPES OF CANCER PAIN

Cancer pain is categorized as somatic, visceral, or neuropathic in origin (2). Somatic pain occurs as a result of the activation of nociceptors in cutaneous and deep tissues. Somatic pain is typically constant and well localized and is frequently described as aching, throbbing, or gnawing. Both bone metastasis and mucosal injury produce somatic pain.

Visceral pain originates from injury to sympathetically innervated organs. Mechanisms of visceral pain include necrosis, ischemia of visceral muscle, serosal or mucosal irritation by algogenic substances, or abnormal distention or contraction of smooth muscle walls within a hollow viscus. The pain is characterized as either dull, deep, and aching or paroxysmal and colicky (2).

Neuropathic pain refers to pain syndromes that occur as a result of nerve injury. Neuropathic pain can occur following surgery or radiation therapy. In addition, certain chemotherapeutic agents (e.g., Taxol, vincristine, vinblastine, and cisplatin) can produce neuropathic pain. This pain is characterized by burning, tingling, and numbing sensations (2).

Pain associated with mucosal injury in the oral cavity can be categorized as somatic (2). Pain associated with mucosal injury in the upper and lower gastrointestinal tract may exhibit the characteristics of visceral pain (2). Research studies are needed, however, that describe the multiple dimensions and more accurately characterize the types of pain that occur with mucosal injury in the oral cavity as well as in the upper and lower gastrointestinal tract. One possible method of accomplishing this would be to have patients describe their pain using the sensory and affective descriptors from the McGill Pain Questionnaire (3). Categorization of the sensory and affective dimensions of the pain associated with mucosal injury would demonstrate which pain dimension is more relevant to patients over time and would also allow researchers to determine which pain descriptors are associated with different types of cancer treatment or with different locations in the gastrointestinal tract. Characterization of this pain as somatic, visceral, or neuropathic is useful clinically and would be aided by patients' assessment of these dimensions of their pain.

EPIDEMIOLOGY OF PAIN ASSOCIATED WITH MUCOSAL INJURY

Pain associated with mucosal injury can occur during chemotherapy, radiation therapy, or bone marrow transplantation. Oral pain associated with stomatotoxic chemotherapy is prevalent in from 40% to 70% of all patients (4). In patients who receive radiation therapy to the head and neck region, as many as 100% will experience pain that increases in severity during the course of treatment and persists after the treatment (5-8). Several studies (9-11) have documented that oral pain associated with bone marrow transplantation is prevalent in from 60% to 85% of all patients. More detailed studies are needed, however, to describe the patterns of oral and rectal pain associated with cancer treatment and to determine whether pain persists following treatment.

Concerning primary or adjuvant chemotherapy, detailed longitudinal studies are needed to determine the patterns of pain intensity associated with various chemotherapy regimens. Information is needed on the intensity and characteristics of pain associated with the initial course of chemotherapy as well as with subsequent courses of chemotherapy.

Radiation therapy to the head and neck region is associated with mucosal injury and pain. The temporal development of radiation-induced pain and the effects of pain on the activities of daily living were evaluated in a study of 14 patients (12) who underwent radiation therapy for head and neck cancer. All patients developed painful mucositis that began during the second

Correspondence to: Christine Miaskowski, R.N., Ph.D., F.A.A.N., Department of Physiological Nursing, University of California, 2 Koret Way, Box 0610, N631Y, San Francisco, CA 94143-0610 (e-mail: chris.miaskowski@nursing.ucsf.edu).

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or third week of radiation therapy. Despite the use of analgesics and anesthetics, the pain experienced by patients was rated as moderate or severe on 37% of the treatment days and was noted to be constant or present throughout most of the day on 58% of the treatment days. Eating and sleep disturbances associated with pain occurred on 55% and 34% of the treatment days, respectively.

Patients who undergo bone marrow transplantation receive high doses of chemotherapy with or without radiation therapy. Patterns of mucositis and pain were evaluated in a study of 47 patients who underwent bone marrow transplantation with high-dose chemotherapy without total-body irradiation (9). The patients' oral cavities were assessed daily, from 9 days before transplant through 21 days after transplant. The patients' oral cavities were assessed for the extent and severity of mucositis; patients reported oral pain by using the short form of the McGill Pain Questionnaire (13). Eighty-nine percent of the patients developed oral mucositis, which began an average of 3 days after transplant, lasted 9.5 days, and was resolved by 12.6 days after transplant. Eighty-six percent of the patients reported oral pain that began an average of 4.5 days after transplant, lasted 6.4 days, and was resolved by 11 days after transplant.

The epidemiology of pain associated with mucosal injury in the oral cavity and in the upper and lower gastrointestinal tract needs to be examined in more detail. Longitudinal studies are needed that evaluate the characteristics, location, and severity of pain associated with all somatotoxic cancer treatment modalities. Also, consideration needs to be given to the scales that are used to measure the severity of the pain that occurs with mucosal injury. Traditional numeric rating scales ranging from 0 (no pain) to 10 (worst pain imaginable) may not be appropriate when evaluating a pain problem that escalates over the course of treatment and for which patients have no prior concept. In these cases, the risk is that the scale's ceiling will be approached well before the highest level of pain is reached unless patients are told that they can use a number higher than 10 if their pain exceeds their previous threshold of "worst pain imaginable." In addition, studies of therapies that are directed at preventing or treating mucosal injury associated with cancer treatment should measure multiple dimensions of the pain experience, including pain intensity and pain relief (i.e., from 0 = no relief to 100 = complete relief) as part of any outcome evaluation of the effectiveness of these therapies.

BIOLOGY OF MUCOSAL PAIN

Neuroanatomic Considerations

Peripheral nerves that respond to noxious stimuli are best characterized in the skin because there they are more accessible for electrophysiologic studies. Those nerves that respond preferentially to noxious stimuli are termed nociceptors. Nociceptors are classified on the basis of the following criteria: conduction velocity, specific response characteristics, presence or absence of a myelin sheath, and modalities of stimulation that evoke a response. On the basis of these criteria, two main classes of cutaneous nociceptors have been characterized: 1) A δ high-threshold mechanoreceptors are myelinated nociceptors that respond with higher discharge frequencies and provide more discriminative information to the central nervous system, and 2) C-polymodal nociceptors are unmyelinated fibers that provide a more diffuse response to noxious stimulation (14,15).

Only one study (16) was found that described the characteristics of mucosal nociceptors in the oral cavity of the rat. This *in vitro*, jaw-nerve preparation of the adult rat evaluated nociceptors in the oral mucosa of the lower jaw by recording activities from single fibers in the lingual nerve. Responses were recorded from a total of 124 single fibers. Fifty-seven percent of the fibers were classified as non-nociceptive. The remaining 67 fibers were characterized by using mechanical (i.e., calibrated von Frey hairs), heat (up to 50°C), and chemical (i.e., bradykinin) stimuli.

Four types of oral mucosal nociceptors were found: 11 A δ high-threshold mechanoreceptors, seven A δ mechanoheat receptors, 21 A δ polymodal nociceptors, and 28 C-polymodal nociceptors. The majority of the nociceptors in the rat's oral mucosa were of the polymodal type. The major characteristics of the nociceptors in the oral mucosa are summarized in Table 1. The fact that the oral mucosa consists mainly of polymodal nociceptors seems to make it functionally suitable for detecting the characteristics of ingested food or drink, which provide various chemical, mechanical, and thermal stimuli to the oral mucosa (16). However, because of the paucity of the research in this area, additional studies are warranted to characterize the various types of nociceptors in the oral mucosa of different animal species. In addition, the nociceptors in the upper and lower gastrointestinal tract need to be enumerated and characterized.

Mechanistic Considerations

The biology of mucosal pain can be explained by using a model of tissue injury (Fig. 1). Injury to the oral mucosa in patients with cancer occurs as a result of the systemic administration of stomatotoxic chemotherapy or the administration of ionizing radiation to the head and neck region or to the upper or lower gastrointestinal tract (17). While the biology of mucosal pain has not been characterized, the mechanisms of cutaneous pain associated with tissue damage and inflammation are well described [for review, see (15)] and are summarized here.

Tissue injury initiates the process of inflammation, characterized by pain, heat, redness, swelling, and loss of function. Pain occurs because primary afferent nociceptors are activated by or become sensitized to noxious stimuli. During inflammation, nociceptors exhibit a lower threshold for stimulation-induced pain or an increased responsiveness to noxious stimuli. The clinical correlate of sensitization is hyperalgesia, when the patient reports "tenderness" at the site of inflammation.

Table 1. Characteristics of nociceptors in the oral mucosa of the rat*

- The receptive field of the polymodal nociceptors in the oral mucosa is statistically significantly larger than that of the A δ high-threshold mechanoreceptors or the A δ mechanoheat receptors.
- The C-polymodal nociceptor in the oral mucosa has the largest receptive field.
- No statistically significant differences were found in the threshold for von Frey stimulation among the four types of nociceptors in the oral mucosa.
- The heat threshold of A δ polymodal nociceptors was statistically significantly lower than that of the other three types of nociceptors found in the oral mucosa.
- The heat threshold of the oral mucosa was similar to that found in the skin.
- The threshold for von Frey stimulation was higher in the oral mucosa than in the skin.
- The oral mucosa is richly supplied with both A δ - and C-innervated polymodal nociceptors.

*Data from reference (16).

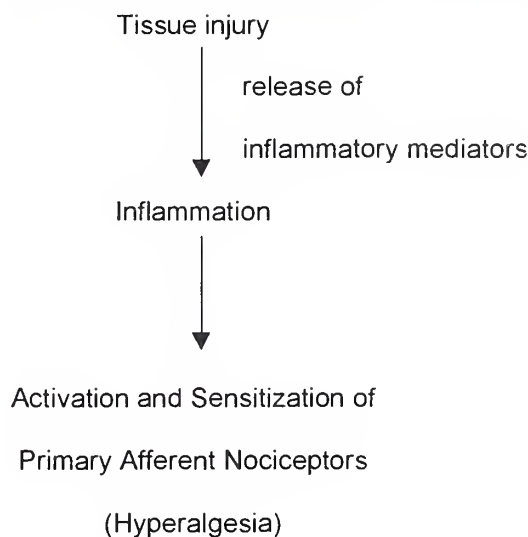


Fig. 1. Peripheral pain mechanisms.

The process of inflammation involves a cascade of events that ensures a rapid physiologic response to tissue injury. Inflammation occurs through the release of a series of chemical mediators. These inflammatory mediators produce pain through direct and indirect effects on nociceptors. The direct effects of inflammatory mediators on nociceptors include activation (i.e., inducing activity in nociceptors) and sensitization (i.e., increasing nociceptor responses evoked by other stimuli) (15).

The inflammatory mediators that directly activate and sensitize primary afferent nociceptors are listed in Table 2. Bradykinin activates primary afferent fibers *in vivo* (18,19) and produces pain in humans. Serotonin is released from activated platelets and is elevated in inflammatory exudates. It activates primary afferent neurons and results in pain in humans (20–24). Glutamate is an excitatory amino acid that contributes to inflammatory hyperalgesia (25–27) and appears to act through the activation of peripheral N-methyl-D-aspartate receptors. An acidic pH produces pain in normal tissues (28,29) and a pH as low as 5.4 was reported in inflamed tissues (30).

The inflammatory mediators that directly sensitize primary afferent nociceptors include prostaglandins, 8(R),15(S)-dihydroxyecosatetraenoic acid [8(R),15(S)-diHETE], serotonin, noradrenaline, adenosine, adenosine 5'-triphosphate, nitric ox-

ide, and nerve growth factor (15). Prostaglandins are the best characterized sensitizing agents. During the process of inflammation, they are synthesized from the arachidonic acid that is released from membrane phospholipids. Arachidonic acid is metabolized to prostaglandins by the cyclooxygenase (COX) pathway (31,32). Prostaglandins decrease nociceptive thresholds in behavioral tests in rodents (33,34) and produce tenderness in humans (35).

The lipoxygenase pathway is the second major pathway involved in the sensitization of primary afferent nociceptors. The lipoxygenase pathway converts arachidonic acid into 8(R),15(S)-diHETE. This leukotriene appears to act directly at the receptor of the primary afferent and results in sensitization of the neuron (33,36). In addition to its direct activation effects, serotonin produces hyperalgesia by acting at a different receptor than the receptor involved in activation (37). The catecholamine noradrenaline has been reported to produce hyperalgesia, but only in the presence of tissue injury (38). Adenosine, adenosine 5'-triphosphate, and nitric oxide appear to exert nociceptive effects in inflamed tissue. Nerve growth factor appears to produce sensitization of primary afferent nociceptors by altering their pattern of gene expression of messenger ribonucleic acid encoding a large variety of peptides that alter the excitability of the neuron including bradykinin receptors and sodium channels (15).

As Sonis (17) points out, "a number of clinical observations suggest a physiological complexity in the development of mucositis." The development of mucositis involves four phases: an inflammatory/vascular phase, an epithelial phase, an ulcerative/bacteriologic phase, and a healing phase (17). Undoubtedly, each of these phases of mucosal injury is associated with some level of pain. The exact mechanisms of pain during each phase of mucosal injury, however, remain to be elucidated. The current article extrapolates information on the nociceptive processes that are known to occur in cutaneous tissues during acute inflammation and suggests that these same processes may be involved in the inflammatory phase of mucosal injury associated with cancer chemotherapy and radiation therapy. Undoubtedly, some of the same inflammatory mediators are involved in producing pain in primary afferents located in the oral mucosa during the initial phase of mucositis. The nociceptive processes that occur during the other phases of mucositis remain to be elucidated not only in the oral mucosa but also in the upper and lower gastrointestinal tract.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Basic and clinical research studies are needed in order to characterize the biology of pain associated with cancer treatment-related mucosal injury. Rigorous longitudinal studies are needed to determine the patterns and severity of pain associated with various stomatotoxic chemotherapy regimens and radiation therapy protocols. Investigations need to characterize the types of pain that occur as a result of mucosal injury not only in the oral cavity but also throughout the entire gastrointestinal tract. The epidemiology of chronic pain and associated side effects should be evaluated in patients who have sustained mucosal injury as a result of cancer chemotherapy or radiation therapy. Finally, the mechanisms of pain experienced during the four phases of mucositis (17) need to be elucidated.

Animal models need to be developed that will allow for an evaluation of the anatomy and physiology of nociceptive pro-

Table 2. Inflammatory mediators that activate and sensitize primary afferent nociceptors

Mediators that activate primary afferent nociceptors	
Bradykinin	
Serotonin	
Excitatory amino acids	
Hydrogen ions	
Mediators that sensitize primary afferent nociceptors	
Prostaglandins	
8(R),15(S)-dihydroxyecosatetraenoic acid	
Serotonin	
Noradrenaline	
Adenosine	
Adenosine 5'-triphosphate	
Nitric oxide	
Nerve growth factor	

cesses in both normal and inflamed mucosal tissues. Emphasis needs to be placed on determining which inflammatory mediators activate and sensitize primary afferent nociceptors during mucosal injury. Knowledge gained from animal models on the mechanisms of pain associated with mucosal injury can be used to develop and test novel therapies to decrease the pain associated with this major clinical problem.

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Mucosal Drug Delivery

Vincent H. L. Lee

This review focuses on epithelial drug transport mechanisms in mucosal drug delivery: the final step of a four-part process. Reference is made to the mucosae lining the oral cavity and the gastrointestinal tract, the two mucosae most often succumbing to the side effects of cytotoxic chemotherapeutic drugs. This review will be devoted to carrier-mediated transport, particularly as it relates to the intestinal dipeptide transporter PepT1. This transporter protein appears to be enriched in tumor epithelial cells, to be rather robust to the cytotoxic effects of chemotherapeutic drugs, and to lend itself to the molecular engineering of drugs that target this transporter in tumor epithelial cells. In contrast to the gastrointestinal tract, much less is known about the type and capacity of drug transport processes in the buccal epithelial cells and about how these processes may be altered in disease state (including cancer) and be manipulated pharmaceutically to optimize drug absorption. [J Natl Cancer Inst Monogr 2001;29:41-4]

Mucosal drug delivery is a multistep process. It comprises (a) targeting of the delivery system at a specific region or cell type in a mucosa, (b) retention of that delivery system where it is anchored, (c) drug release from the delivery system at a predetermined pattern that is not necessarily constant, and (d) access of the drug to the drug-transport machinery in the epithelial cells. In the context of this conference, I will focus on the gastrointestinal tract and the oral cavity, the two mucosae that often succumbed to the side effects of chemotherapy. While targeting drug release to either the small or the large intestine can be achieved by exploiting the broad biochemical differences between those two regions in the gastrointestinal tract, such as pH and microbial enzyme content (1,2), we do not yet know the unique biochemical markers in each of the three parts of the small intestine to target drug release at a given part of this organ. The same can be said of the large intestine. In contrast, targeting drug release to a specific region in the oral cavity is relatively straightforward, given its accessibility. In light of the differences among the buccal, sublingual, gingival, and palatal tissues in the oral cavity, as shown in Table 1, it is expected that drug bioavailability would vary with the tissue of exposure.

EPITHELIAL DRUG TRANSPORT MECHANISMS

However sophisticated the drug delivery system is with respect to targeting, retention, and pattern of drug release, there must be a match between the drug's physicochemical characteristics and the epithelial cell's endogenous transport mechanisms for drug uptake and transport to occur. These mechanisms fall into two categories: passive transport and active transport. Passive transport, in turn, comprises paracellular and transcellular transport, while active transport comprises carrier-mediated transport and endo-transcytosis. In general, the epithelial transport of any drug can be considered as the sum of all four trans-

port processes, although in reality, only one, or at most two, of the four transport mechanisms would predominate. Transforming growth factor- β 3 (TGF- β 3), a 25-kd protein, apparently reduced the severity of oral mucositis induced in a hamster model (3) sufficiently enough to implicate a role of endocytosis in protein uptake by the buccal epithelial cells. Nevertheless, the possibility that this protein does not need to enter the cells to bring about a pharmacologic effect cannot be ruled out. In the study just cited, recombinant TGF- β 3 (20 μ g) was applied topically to the hamster cheek pouch as a 7.9- μ M solution in 0.1 mL physiologic-buffered saline containing 0.01% Tween 20 and 1% ethanol four times daily over a 24-hour period before chemotherapy with an intraperitoneal dose of 5-fluorouracil (5-FU) at doses of 60–80 mg/kg on day 0 and 40–60 mg/kg on day 2.

THE CASE FOR CARRIER-MEDIATED TRANSPORT

Carrier-mediated transport is an area of active research in drug delivery today. Carrier-mediated transport, either Na⁺- or H⁺-coupled, may be playing a more prominent role in drug transport than originally envisaged (4). For instance, benzoic acid, a weak acid that is predicted to be absorbed exclusively by transcellular passive transport in accordance with the pH-partition hypothesis, actually mainly relies on the H⁺-monocarboxylate transporter for absorption (5). The associated K_m is 1.28 mM. It is interesting that a monocarboxylate transport system may also exist in cultured buccal epithelial cells in the rabbit (6). Utoguchi et al. (7) subsequently verified the involvement of carrier-mediated transport in the uptake of salicylic acid from the hamster cheek pouch, followed by drug appearance in the systemic circulation. This finding sets the stage for searching for other drug transporters in the buccal epithelial cells that may be of utility in facilitating drug uptake.

THE INTESTINAL DIPEPTIDE TRANSPORTER PEPT1 AS AN ILLUSTRATIVE EXAMPLE

The intestinal dipeptide transporter PepT1 is perhaps the drug transporter that has captured the most attention toward mucosal drug delivery recently. This H⁺-coupled transporter has a rather wide substrate specificity (8–15), including dipeptides and tripeptides, β -lactam antibiotics, angiotensin-converting enzyme inhibitors, and bestatin and even compounds without an obvious peptide bond or equivalent, such as δ -aminolevulinic acid (16) and ω -amino fatty acids (ω -AFA) (17). In the small intestine, the population of this transporter increases from the duodenum to the ileum (18).

Correspondence to: Vincent H. L. Lee, Ph.D., Department of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Ave., PSC 708, Los Angeles, CA 90089-9121 (e-mail: vincentL@hsc.usc.edu).

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Table 1. Oral epithelium characteristics*

Tissue	Structure	Thickness, μm^\dagger	Blood flow, $\text{mL min}^{-1} \text{cm}^{-2}\ddagger$
Buccal	Nonkeratinized	500–600	2.40
Sublingual	Nonkeratinized	100–200	0.97
Gingival	Keratinized	200	1.47
Palatal	Keratinized	250	0.89

*Adapted from (31).

 † Thickness of human oral epithelium (32). ‡ Blood flow in oral mucosa of the rhesus monkey (33).

Enrichment in Tumor Epithelial Cells

Nakanishi et al. (19) and Gonzalez et al. (20) demonstrated independently that PepT1 appeared to be enriched in cancer epithelial cells. Specifically, Nakanishi et al. (19) observed [^{14}C]Gly-Sar uptake by the human fibrosarcoma cell line HT1080 but not by IMR-90 (a normal diploid cell line), with a K_m of $11.4 \pm 3.3 \text{ mM}$ and a V_{\max} of $26.8 \pm 4.0 \text{ nmol/15 minutes}$ per milligram protein. Optimal dipeptide uptake was at pH 6 and was subject to competition by cefadroxil and bestatin. Similar observations were made by Gonzalez et al. (20) in two human pancreatic cancer cell lines—AsPc-1 and Capan-2. The K_m was $0.80 \pm 0.17 \text{ mM}$ for AsPc-1 and $1.0 \pm 0.2 \text{ mM}$ for Capan-2, and the corresponding V_{\max} was $65 \pm 4.4 \text{ nmol/10 minutes}$ per milligram protein and $10.9 \pm 1.0 \text{ nmol/10 minutes}$ per milligram protein, respectively. The enrichment of PepT1 in cancerous epithelial cells represents a target of drug design for the specific delivery of peptidomimetic anticancer drugs into tumor cells. Such a strategy has been applied to improving the intestinal uptake of the nucleoside analogues acyclovir and zidovudine (AZT) by forming 5'-amino acid ester prodrugs (Fig. 1) (21). There was a threefold to 10-fold increase in intestinal permeability that was selective for the L-amino acid esters.

Resistance to Cytotoxic Effects of Chemotherapy

For reasons that are not immediately forthcoming, PepT1 appears to be more resilient to the cytotoxic effect of chemotherapy than do other membrane-bound proteins. Tanaka et al. (18) investigated the mechanism of the resistance of PepT1 to mucosal injury in the intestine of rats treated with an oral dose of 300 mg/kg 5-FU. These investigators attributed such a resistance to increased synthesis of PepT1 rather than to a change in the kinetic properties of the residual absorbing cells. Specifically, although the amount of sucrase and a Na^+ -dependent glucose transporter protein in intestinal vesicles decreased markedly after 5-FU treatment, the amount of PepT1 protein remained largely unaffected. Moreover, levels of amino acid, glucose, and phosphate transporter messenger RNAs (mRNAs) were profoundly depressed in 5-FU-treated animals, whereas the level of PepT1 mRNA conversely increased. Coincidentally, Ihara et al. (22) reported that PepT1 gene regulation was substantially enhanced under malnourishment in spite of atrophic changes of intestinal mucosae in Sprague-Dawley rats.

Subcellular Compartmentalization and Its Modulation

The apical plasma membrane is not the only subcellular component in which PepT1 is found. PepT1 also exists in the lysosomal membrane (23–25) (Fig. 2). This subcellular compart-

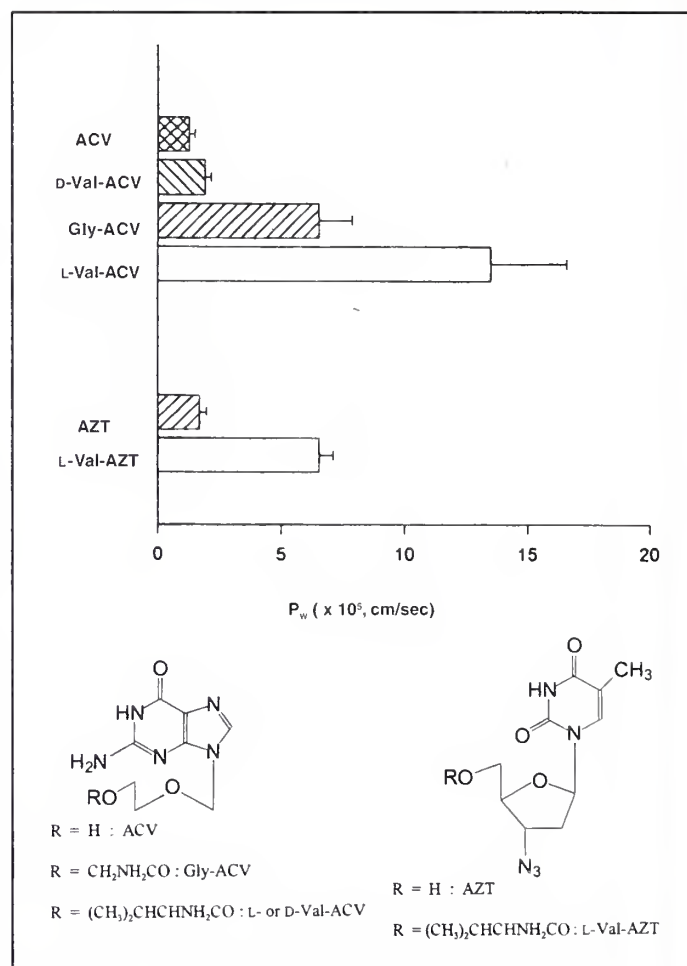


Fig. 1. Chemical structures of acyclovir (ACV) and zidovudine (AZT) and their intestinal membrane permeabilities in comparison with their prodrugs in rats. Error bars denote mean \pm standard error of the mean for $n = 4-6$. Adapted from (21).

mentalization of PepT1 raises the questions of whether the ratio of PepT1 population between the lysosomal and apical plasma membrane is static, how this ratio may be altered pharmacologically, and what role biopolymers may play in altering this ratio. We do not yet have information on whether the compartmentalization of PepT1 in the intestinal epithelial cells is static. Nevertheless, there is evidence for the pharmacologic alteration in the density of PepT1 at the apical plasma membrane.

The acute translocation of PepT1 from the intracellular PepT1 pool to the apical surface was reported by Thamotharan et al. (26) in 1999. They found that preincubation of Caco-2 cells with 5 nM insulin for 1 hour stimulated Gly-Gln uptake by 80% (Fig. 3), consistent with an elevation of the apical expression of PepT1 by the same magnitude. This effect manifested itself within 60 minutes. There was no change in the mRNA level of PepT1. Moreover, disruption of the *trans*-Golgi network (TGN) with 5 μM brefeldin A, thereby halting the migration of newly synthesized PepT1 to the apical membrane, did not affect either the basal or insulin-stimulated dipeptide uptake. In contrast, 10 μM colchicine, which depolarized microtubules (MTs), abolished insulin-stimulated dipeptide uptake, even though it did not have any effect on basal dipeptide uptake. This finding suggests that insulin may stimulate the translocation of PepT1 to cell surface in an MT-dependent manner.

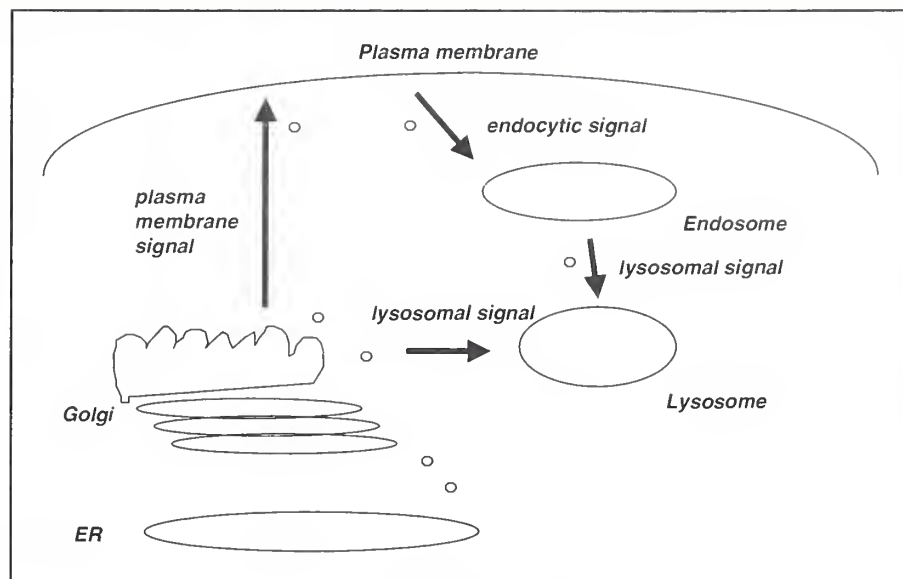


Fig. 2. Trafficking pathways of PepT1 in an epithelial cell. Pathway 1 denotes recruitment; pathway denotes retrieval of the transporter protein. Adapted from (25). ER = endoplasmic reticulum.

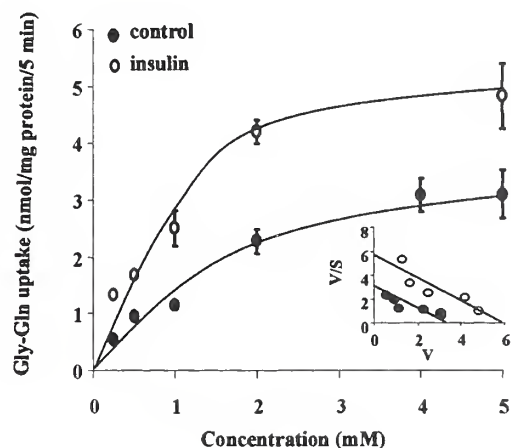


Fig. 3. Effect of 5 nM insulin on kinetics of Gly-Gln uptake in Caco-2 cells. Error bars denote mean \pm standard error of the mean for $n = 3-4$. Inset: Eadie-Hofstee plots of Gly-Gln uptake, where V is rate of uptake (nmol/mg protein per 5 minutes) and S is substrate concentration (mM). Adapted from (26).

As another example of pharmacologic manipulation of the ratio of PepT1 in the apical plasma membrane and the intracellular pool, Fujita et al. (27) recently reported that a selective σ_1 ligand, (+)pentazocine, increased the uptake of Gly-Sar in Caco-2 cells in a concentration-dependent (0.001–10 μ M) and time-dependent (1–24 hours) manner. A minimum of 2 hours of incubation was required, and the maximal increase in dipeptide uptake was 200%. Kinetically, this can be attributed entirely to an increase in the maximum velocity of dipeptide uptake. Semi-quantitative reverse transcription–polymerase chain reaction suggests that (+)pentazocine up-regulates PepT1 in Caco-2 cells at the level of increased mRNA.

As an alternative to the use of drugs to manipulate the density of PepT1 at the apical plasma membrane, biopolymers may be considered. Such a speculative role for biopolymers is based on the observations that chitosan, as well as its derivative, enhances paracellular permeability in Caco-2 cells (28) and that chitosan

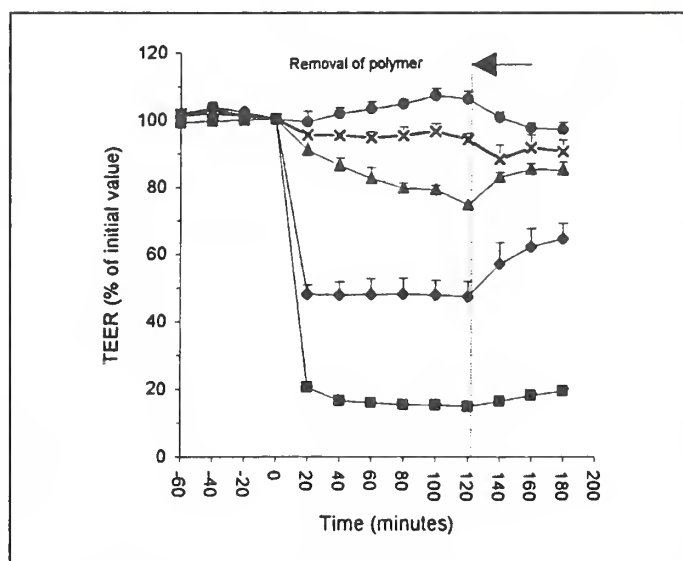


Fig. 4. Effect of *N*-trimethylchitosan (TMC) on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers. Error bars denote mean \pm standard deviation for $n = 3$. Key: Control (x), TMC 1% (filled circles), TMC 1.5% (filled triangles), TMC 2% (filled diamonds), and TMC 2.5% (filled squares). Dotted line represents start of reversibility experiment. Adapted from (30).

enhances the transcytotic capacity of Calu-3 cells (29). As can be seen in Fig. 4, incubation of Caco-2 cell monolayers with *N*-trimethylchitosan (TMC), a water-soluble chitosan derivative, resulted in pronounced and immediate reduction in transepithelial electrical resistance (TEER) in a concentration-dependent manner over the 1.5%–2.5% range (30). Concentrations of 1% or less were ineffective. Reversibility of the TEER-lowering effect was evident at 1.5% and 2% on removing TMC from making contact with the cell monolayer. Monolayer exposed to 2.5% TMC was slower in recovery. Using a human airway epithelial cell line (Calu-3), Witschi and Mrsny (29) found that spray-dried chitosan microspheres (14%–17% acetylation, molecular weight 300 000), 2–4 μ m in diameter, enhanced the transport of bovine serum albumin by 20 times over a 6-hour period. Induction of the release of cytokines, such as interleukin

(IL)-6 and IL-8, was suggested to be the triggering factor. It would be interesting to evaluate whether chitosan also affects the trafficking of membrane-bound transporter proteins such as PepT1.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Clearly, there is effective transport machinery in the intestinal epithelial cells to facilitate the absorption of a diverse array of therapeutic molecules. By contrast, much less is known about the basal capacity of the various transport processes in the epithelial cells lining the various regions of the oral cavity and about how this capacity may be altered during oral mucositis. This subject deserves further study. It is equally important to investigate how these transport processes in both epithelia may be altered in patients undergoing chemotherapy and to determine whether such possible alteration may be exploited to protect those epithelial cells from further insult with the use of chemoprotective drugs that would seize on an altered transport pathway.

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NOTE

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Mucositis in Head and Neck Cancer: Economic and Quality-of-Life Outcomes

Amy Peterman, David Cella, Gerald Glandon, Deborah Dobrez, Susan Yount

Outcomes research typically assesses three major health care outcomes, including quantity of life, quality of life (QOL), and health care cost. This article highlights the impact of treatment-associated mucositis on health care costs and QOL. After a background description of the economic analyses of overall cancer treatment costs and of the incremental costs associated with other treatment side effects, data from a retrospective study of mucositis-specific costs are presented. The second half of this article reviews current knowledge about the effect that mucositis has on QOL. Because the empirical work that specifically evaluates mucositis and QOL is quite limited, studies examining proxies for mucositis grading are described. These include studies comparing the QOL of patients currently undergoing treatment, in which symptoms likely to be associated with mucositis are worse, with that of patients who have completed treatment. Also discussed are investigations examining both the relationship between specific mucositis-associated symptoms, such as pain and difficulty swallowing, and QOL and the weighting of different domains of mucositis-associated problems. Finally, several future research directions are suggested, with the intent of expanding knowledge about the economic and QOL impact of mucositis in patients treated for head and neck cancer. [J Natl Cancer Inst Monogr 2001;29:45-51]

With the increasing use of aggressive chemotherapy and/or radiation therapy protocols to treat head and neck cancer, attention to the consequences of the major treatment side effect, mucositis, is clearly warranted. This article will highlight the impact of mucositis on health care costs and quality of life (QOL).

ECONOMIC COSTS OF MUCOSITIS

Economists divide the economic costs of illness into three basic categories. These categories include direct costs, which can be both medical (e.g., hospital admissions and prescription medications) and nonmedical (e.g., transportation and health care supplies); indirect costs such as lost wages from time away from work; and intangible costs, which are largely psychosocial in nature (1). Economic analyses are regularly included in clinical trials (2), and the analyses typically examine all direct medical costs associated with a particular treatment. The absolute costs of two treatments can then be compared. In addition, a cost-effectiveness ratio, or the ratio of economic costs to effectiveness or value, may be calculated.

Economic cost comparisons of different treatment modalities for head and neck cancer have been conducted. For example, Myers et al. (3) evaluated three potential treatments for stage T1 glottic larynx cancer: microlaryngoscopy, radiation therapy, and hemilaryngectomy. The results indicated that microlaryngoscopy had the lowest cost, followed by radiation therapy, with hemilaryngectomy being the most expensive. In a cost-effective-

ness analysis examining actual costs, not charges, for treatment of stage I or II glottic cancer, Foote et al. (4) demonstrated that the costs of transoral endoscopic removal were lower than those of radiation therapy and partial vertical laryngectomy. However, the cost-effectiveness portion of the analysis attempted to take into account incidence of local recurrence and voice quality: The inclusion of these factors, which the authors judged to be superior in the radiation therapy group, led to the conclusion that radiation therapy may provide the best value for a moderate incremental cost. Other investigators (5,6) have examined the relationship of particular diagnostic and staging procedures to treatment costs, including positron emission tomography scans and fine-needle aspiration biopsies. Sherman et al. (7) provided a recent review of economic analyses in head and neck cancer, including the methodologic challenges inherent in conducting such investigations with a very heterogeneous population.

Less common are evaluations of the economic costs of a particular treatment side effect, i.e., an evaluation of the costs associated with managing a particular symptom, such as mucositis, that arises as a consequence of cancer treatment. Such incremental costs are those attributable to mucositis over and above those attributable to the cancer and its treatment.

While there appear to be no published investigations of the costs of treating mucositis in head and neck cancer, the incremental costs associated with the treatment of other toxic effects have been examined—most notably, febrile neutropenia and nausea/vomiting. For example, McQuaker et al. (8) evaluated the use of filgrastim in patients who had received a stem cell transplant. The authors identified several major costs potentially associated with neutropenia, including intravenous antibiotic therapy, days with fever, and number and cost of inpatient hospital days. In a randomized trial of filgrastim versus placebo, they demonstrated that the economic costs in the filgrastim group were statistically significantly lower than those in the placebo group, based on the identified cost items.

A somewhat similar method was used by Stewart et al. (9) in their investigation of the costs of preventing and treating nausea and vomiting in patients receiving highly emetogenic chemotherapy. Specific inpatient and outpatient resources were enumerated, including those for different antiemetic drugs; supplies; nursing, physician, and pharmacist time; and outpatient and inpatient hospital visits/stays. The cost associated with each resource was then specified. Finally, costs were calculated for patients receiving ondansetron as opposed to other antiemetic regimens. Results strongly favored ondansetron, with statisti-

Affiliations of authors: A. Peterman, D. Cella, D. Dobrez, S. Yount, Northwestern University, Evanston, IL; G. Glandon, University of Alabama, Birmingham.

Correspondence to: Amy Peterman, Ph.D., Center on Outcomes, Research and Education, 1000 Central St., Suite 101, Evanston, IL 60201 (e-mail: a-peterman@northwestern.edu).

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cally significantly lower economic costs primarily because of fewer inpatient stays for gastrointestinal complications.

In summary, evaluations of economic costs and cost-effectiveness of cancer treatments have become increasingly more common, although little of this work has been done in head and neck cancer. Investigations typically target either the overall costs associated with a particular cancer treatment or the specific costs believed to be associated with a treatment side effect.

In light of the prevalence and severity of mucositis in patients treated for head and neck cancer and the lack of information on the incremental economic costs associated with it, we recently evaluated the direct medical costs associated with mucositis management. The study consisted of three parts. First, a focus group of physician and nurse experts in head and neck cancer treatment met to identify the potential resources used in the management of mucositis. Second, a retrospective chart review was conducted in a consecutive sample of 45 patients treated with radiation therapy or combined chemoradiotherapy for head and neck cancer at a single institution. Resources specifically aimed at mucositis management were identified for each patient throughout the course of his or her treatment. Finally, we utilized two costing methodologies to derive monetary cost estimates for the mucositis management resources used.

The focus group consisted of two physicians (one radiation oncologist and one medical oncologist) and two nurses, all of whom had at least 3 years' experience treating head and neck cancer patients. They identified five categories of resource use for mucositis management that would account for incremental direct medical costs. These resources were primarily directed at managing pain and nutritional/hydration consequences and included hospitalization for dehydration, malnourishment, or pain; additional professional time spent by physicians, nurses, and nutritionists in mucositis management; maintenance of appropriate nutritional and hydration status, such as placement of gastrostomy tubes, intravenous (IV) hydration, and nutritional supplements; prescription medications for pain and other mouth care; and home health assistance when needed for tube feedings and IV hydration.

We then reviewed the charts of a consecutive series of patients treated with radiation therapy or with chemoradiotherapy protocols for head and neck cancer at a single institution from May 1994 through December 1996. Subjects were eligible if they received their complete course of cancer treatment at this institution, so that necessary records were available for review, and if they had not received previous radiation therapy or chemotherapy for head and neck cancer. Charts of the 45 patients who met these eligibility criteria were reviewed, and data on basic demographic, disease, and treatment information; number of visits to health care personnel; hospitalizations during treatment; the use of prescription medications, nutritional supplements, and IV hydration; and mucositis severity throughout treatment were recorded for the period beginning with the start of treatment and ending with the date 2 months after the end of treatment, at which time most of the treatment-related mucositis had remitted.

One research assistant reviewed all records, and 10% of the records were also reviewed by the first author. The interrater agreement on coding was calculated to be an acceptable 85%. The main area of nonagreement was in the number of professional visits, and this appeared to be attributable to the multiple sections of the charts where visits could be recorded (i.e., prog-

ress notes, treatment log, flow sheet). The noted discrepancies were corrected through rereview of the pertinent charts.

The median age of the subjects was 59.3 years (range, 33–87 years). The subjects were mostly male (66.7%) and European-American (65.1%), with a range of disease severity (stage I: 14.3%; stage II: 16.7%; stage III: 26.2%; and stage IV: 42.9%). Fifty-six percent were treated with radiation therapy only, while 13% were treated with alternating chemotherapy and radiation therapy, and 31% were treated with concurrent chemotherapy and radiation therapy.

Tables 1 and 2 show the mucositis-associated resources used by this sample of patients. Table 1 demonstrates that roughly one third of the patients had substantial nutritional or hydration support needs, 15.6% of which were severe enough to require hospitalization for dehydration. Table 1 also displays professional time and the portion of this time calculated to be incremental to the management of mucositis. That is, for the resource-use categories other than professional time, all resources were assumed to be incremental to mucositis (i.e., it is assumed that the need for pain medication or IV hydration resulted directly from the presence and severity of mucositis). Professional time, however, differs in that some nurse and physician visits would be a regular part of patient care during head and neck cancer treatment, while others might be extra visits occasioned by mucositis-associated problems. Because the reason for each professional visit was not available in this retrospective review, we had to make an assumption that subjects with more severe mucositis would have more professional visits prompted by the side effects than would those with less severe mucositis. We calculated incremental professional time by subtracting the mean number of visits for those with less severe (grades 0 or 1) mucositis from the mean number for those with more severe (grades 2–4) mucositis. The drawback of this assumption is that it assigns all extra visits to mucositis, although subjects with more severe mucositis also had other more severe problems or side effects that might have

Table 1. Mucositis resource use (n = 45)

	Mean No. (SD)	%/Range
Hospitalization		
G-tube placed		
Yes	16	35.6%
No	29	64.4%
G-tube replaced because of complications		
Yes	3	6.7%
No	42	93.3%
Hospitalized for hydration		
Yes	7	15.6%
No	38	84.4%
Professional time		
Physician visits	11.4 (3.9)	5–22
Nurse visits	17.4 (8.8)	5–38
Incremental professional time		
Physician visits	3.0 (2.9)	0–12
Nurse visits	7.7 (7.9)	0–25
Outpatient support		
Nutritionist visits	3.2 (2.01)	0–7
Intravenous hydration		
Required	13	28.9%
Not required	32	71.1%
Total No. of liters required	2.8 (9.1)	0–54
Nutritional supplements		
Required	37	82.2%
Not required	8	17.8%
Total No. of cans of supplement	235.2 (255.2)	0–973

Table 2. Prescription drug usage (n = 45)

Prescription drug	Not prescribed	One course prescribed*	Two courses prescribed	Three courses prescribed
Advil TM	41	4	0	0
Diflucan TM	34	6	2	3
Dilaudid TM	44	1	0	0
Duragesic/Fentanyl TM patch	40	4	1	0
MS Contin TM	44	1	0	0
Mycelex TM	43	2	0	0
Nystatin TM	37	7	1	0
Peridex TM	43	1	1	0
Roxanol TM	33	9	3	0
Salagen TM	40	4	1	0
Stomatitis cocktail TM	23	17	4	1
Trilisate TM	31	11	2	1
Tylenol 3 TM	20	18	5	2
Tylenol 4 TM	44	1	0	0
Vicodin TM	38	6	0	1

*One course assumed to be 1 month for all medications except Diflucan, for which one course is 7 days.

occasioned the additional professional visits. Thus, this may be an overestimate of the incremental professional time specifically prompted by mucositis. Finally, the relatively common use of narcotic and nonnarcotic pain relievers and antifungal medications is shown in Table 2.

The third step in this study was to assign a dollar value to the opportunity cost of the resource-use categories. Because true cost data are quite difficult to obtain, we used charges and reimbursements as proxies for actual costs. Specifically, we determined low and high estimates of charges/reimbursements for each of the categories. This allowed us to compare the results of two different costing methodologies. For hospitalizations, professional time, and outpatient hydration, Medicare reimbursements were used as the low estimate, and the charges billed by the hospital oncology unit were used as the high estimate. Low and high estimates for nutritional supplements came from a local pharmacy chain price and a hospital-based pharmacy price, respectively. Wholesale and retail prices from the 1996 *Drug Topics Red Book* (10) formed the low and high estimates for prescription medications, while values for the nutritionist time were calculated on the basis of charges for minimal (basic) versus extensive (complicated) visits.

The low and high incremental cost estimates for each resource-use category can be found in Table 3. Note that, as would be expected, hospitalizations add the largest amount to mean total incremental cost (\$1840–\$1966). Outpatient support (\$534–\$828) and prescription medication (\$452–\$1049) have intermediate costs. The cost of incremental professional time is

relatively low (\$122–\$194). Standard deviations are quite large for all category estimates, revealing the strikingly wide variability of mucositis-related costs in this sample.

Finally, we examined whether costs differed between subjects who differed in the degree of severity of the mucositis that developed during treatment. For these analyses, patients were categorized as having either low-severity (grade 0 or 1) or high-severity (grades 2–4) mucositis based on the most severe grade mentioned in the treatment notes. Wilcoxon rank-sum tests were used to test for differences in costs as a function of mucositis severity. At both low and high estimates of costs, the group with more severe mucositis had statistically significantly greater costs in two of the four cost categories, including outpatient nutrition/hydration support (low: $z = 2.06$, $P = .04$; high: $z = 2.22$, $P = .03$) and prescription medications (low: $z = 3.23$, $P = .001$; high: $z = 3.51$, $P = .0005$). The cost owing to hospitalization did not differ statistically significantly by mucositis severity at either the low ($z = 1.59$, ns [not statistically significant]) or high ($z = 1.59$, ns) estimates of cost. However, the mean differences were quite large, with a difference of \$1331 at the low cost estimate and \$1522 at the high cost estimate. It is likely that the lack of statistically significant difference can be attributed to the very large standard deviations of the estimates. Because incremental professional time was defined as the additional increment of time spent with patients with more severe mucositis, it was not possible to calculate a z score for incremental professional time. Total cost estimates did differ between mucositis severity groups at both low ($z = 2.82$, $P = .005$) and high ($z = 3.07$, $P = .0021$) cost estimates.

The results of this study must be considered in light of the following limitations, which were largely a result of the retrospective nature of the study. First, there was not a standard mucositis grading system in use when the data were collected. Thus, variability in the application of a single grading system across providers or across grading systems may have introduced unmeasured error into the results comparing costs between those with low-grade mucositis and those with high-grade mucositis. Second, it was not always possible to ascertain whether a hospitalization for rehydration was needed because of mucositis or because of chemotherapy-induced nausea/vomiting. We made the assumption that all such hospitalizations could be attributed to mucositis. Thus, the estimate of the cost of inpatient care for hydration might be somewhat inflated. Finally, it should be noted that the large standard deviations of the cost estimates might lessen confidence in their accuracy. Further work is clearly needed to address these study limitations.

In summary, despite the constraints imposed by the retrospective nature of this study, the findings indicate that mucositis has

Table 3. Estimates of incremental costs due to mucositis (n = 45)*

Cost category	Low cost		High cost	
	Mean/median (SD)	Range	Mean/median (SD)	Range
Incremental professional time	\$122/\$32 (161)	\$0–\$623	\$194/\$54 (257)	\$0–\$992
Outpatient support†	\$534/\$418 (432)	\$0–\$2069	\$828/\$614 (810)	\$0–\$4418
Prescription medications†	\$452/\$329 (418)	\$0–\$160	\$1049/\$508 (1190)	\$0–\$4563
Hospitalizations	\$1840/\$0 (2765)	\$0–\$12 924	\$1966/\$0 (2945)	\$0–\$13 675
Total costs†	\$2949/\$1281 (3252)	\$0–\$15 472	\$4037/\$2704 (4119)	\$0–\$19 182

*SD = standard deviation.

†Low and high cost estimates are significantly different using the Wilcoxon rank-sum test ($P < .05$).

statistically significant direct medical costs (approximately \$3000 \pm \$1000 per treatment episode) and that these costs are greater for patients experiencing more severe mucositis. A similar type of methodology could be used in a prospective study to provide a more precise estimate of both resource use and associated costs across the course of treatment.

MUCOSITIS AND QUALITY OF LIFE

Health-related QOL "refers to the extent to which one's usual or expected physical, emotional and social well-being are affected by a medical condition or its treatment" (11). The two key aspects of QOL are that it is subjective and multidimensional (12-14). Regarding subjectivity, the field of QOL very clearly emphasizes the centrality of the patient's perspective. The judgments and comparisons between current and expected functioning that make up one's QOL are complex (15) and cannot easily be inferred from outside.

QOL measures typically assess at least four dimensions, including physical, emotional, social, and functional well-being. In brief, physical well-being refers to perceived bodily function or dysfunction, including the level of physical symptoms; emotional well-being includes both positive mood, such as hope and joy, and negative mood, such as depression and anxiety; social well-being is the ability to maintain important social relationships and a feeling of being supported by others; and functional well-being is the ability to perform and enjoy normal daily activities. Other investigators have emphasized additional QOL dimensions, including sexuality (16) and spirituality (17). Total health-related (global) QOL, then, is an aggregation of a number of individual dimensions.

It has been demonstrated that physical and psychological symptoms and/or side effects have a statistically significant impact on the various QOL dimensions (18,19). In our work, we found a strong linear relationship between severity of symptoms, rated from "not at all" to "very much," and scores on individual dimensions of, and total, QOL. Symptoms/side effects evaluated in this sample of 1163 patients with mixed cancer types included pain, trouble sleeping, weakness, nausea, diarrhea, tension, worry, irritability, and depression (18). The fact that findings across a variety of symptoms and toxic effects were similar lends credence to the exploration of the QOL impact of another important side effect: mucositis.

One way to begin to examine the relationship between mucositis and QOL is to consider its possible consequences and how they may affect each of the major QOL dimensions. Thus, major factors associated with mucositis include pain, difficulty swallowing, impaired ability to eat and drink, substantial time needed for complex mouth care regimens, and impaired ability to speak and communicate (20,21). Pain and difficulty swallowing are symptoms that can be considered to be part of physical well-being. Impairment in eating/drinking and speaking/communicating are functional problems that may also have sizeable effects on one's social well-being. That is, meals are often social gatherings, and an inability to participate fully in them may negatively affect feelings about relationships with others. Speech problems and the potential need to find alternative methods of communication for a period of time also likely serve to decrease the ability to participate in social interactions and derive pleasure from them. The requirement to perform complex mouth care regimens may also be considered to be a functional

impairment, since it takes away time and energy that could otherwise be devoted to more enjoyable daily activities. These consequences, separately or in total, may have a substantial impact on emotional well-being as well. That is, sadness, tension, or feelings of isolation and loss of self-identity can result from physical symptoms, impaired functioning, and decreased social interaction.

Although to date no specific instruments have been created to measure the QOL impact of mucositis, there are several well-validated QOL questionnaires for patients with head and neck cancer: all include questions that address possible mucositis-related impairments. Two questionnaires specifically address the functional abilities, or performance status, that may be affected by head and neck cancer and its treatment. These include the List Performance Status Scale for Head and Neck Cancer (PSS-HN) (22) and the University of Washington QOL questionnaire (UWQOL) (23). The PSS-HN is a clinician-rated instrument that consists of three subscales assessing normalcy of diet, understandability of speech, and eating in public. Dysfunction in these three areas is rated on a scale from 0% to 100%, with higher scores indicating fewer problems. The UWQOL is a nine-item scale on which patients rate the level of difficulty experienced in areas such as pain, disfigurement, activity, eating, employment, and speech.

Two other questionnaires widely used to evaluate QOL in head and neck cancer are the European Organization for Research and Treatment of Cancer QLQ-C30 + Head and Neck Module (24) and the Functional Assessment of Cancer Therapy—Head and Neck Cancer Scale (FACT-H&N) (25). Both are composed of a general questionnaire assessing the major QOL dimensions discussed above and an additional subscale addressing head and neck cancer-specific problems that are not contained in the general questionnaire (e.g., "I have pain" could assess mucositis-related pain but is included in the physical well-being subscale and so is not included in the additional concerns subscale). For example, the FACT-H&N contains 27 items assessing physical, social/family, emotional, and functional well-being and an 11-item head and neck cancer subscale. Subscale items are shown in Table 4. Two strengths of this type of general plus specific questionnaire are the potential to calculate a total (general + head and neck cancer specific) QOL score and the possibility of examining the relationship between head and neck cancer-specific concerns and the other QOL dimensions. For example, it would be possible to evaluate whether those with greater head and neck cancer-specific problems also report more impairment in functional or social well-being. In general, there is a high degree of concordance between head and neck cancer-

Table 4. Functional assessment of cancer therapy—head and neck cancer subscale items

I am able to eat the foods that I like.
My mouth is dry.
I have trouble breathing.
My voice has its usual quality and strength.
I am able to eat as much food as I want.
I am unhappy with how my face and neck look.
I can swallow naturally and easily.
I smoke cigarettes or other tobacco products.
I drink alcohol (e.g., beer, wine, etc.).
I am able to communicate with others.
I can eat solid foods.

specific scales, such as the PSS-HN, the UWQOL, and the FACT-H&N subscale, with a lesser association between head and neck cancer-specific scales and the general QOL scales of the FACT-H&N; this suggests an additional perspective that can be gained on patients' QOL by using both general and disease-specific instruments (26).

As can be seen by examining the questions of the disease-specific scales, about half of the questions address concerns that might be specifically related to mucositis (e.g., ability to eat and communicate, swallowing, and voice quality on the FACT-H&N subscale). However, previous surgery and the disease itself are other potential causes of these symptoms. Mucositis is widely acknowledged as a consequence of high-dose chemotherapy (27–29), radiation therapy (21,30,31), and the multimodal treatment regimens used to treat head and neck cancer (32–34). A number of studies (20,21,29–31,34–36) have commented that mucositis adversely affects patients' QOL but have not done so in the context of an empirical investigation of QOL.

In the absence of empirical data, another way to evaluate the importance of mucositis to QOL is to compare QOL scores of those on chemotherapy or radiation treatment, who are, therefore, likely to be experiencing mucositis, with patients not currently receiving treatment. A number of authors have examined this question (25,37–43).

In a series of three studies, List et al. (25,37,38) evaluated treatment-related symptoms and QOL. In a cross-sectional comparison, List et al. (25) demonstrated that patients undergoing treatment reported greater head and neck cancer-specific concerns as well as poorer physical, functional, and total well-being. List et al. (37) also investigated a cross-sectional sample of patients with head and neck cancer (stages II–IV) 1 year after completion of an intensive chemoradiotherapy protocol. Although 70% of this sample experienced mucositis of grade 3 or 4 during treatment, this experience was not long lasting and was not related to their experience of residual pain or eating difficulties 1 year later. Residual mouth and throat pain were, however, related to QOL 1 year after treatment. Subsequently, List et al. (38) conducted a longitudinal study examining QOL in a sample of patients before, during, and after concomitant chemotherapy/radiation treatment. In the comparison of head and neck cancer-specific concerns before and during treatment, there was greater impairment during treatment in almost all areas. Thus, a greater percentage of patients on chemotherapy/radiation treatment reported problems in global functioning, normalcy of diet, and speech on the PSS-HN (22) and were more likely to report such specific symptoms as difficulty swallowing, mouth and throat pain, and hoarse voice. In those patients who survived at least 1 year after treatment, improvements were reported in global functioning, normalcy of diet, swallowing difficulties, and throat pain. Speech, mouth pain, and hoarse voice were not statistically significantly improved from reports during treatment. Scores on the FACT-H&N indicated only an improvement in physical well-being from treatment to 1 year after treatment. These results suggest that mucositis related to chemotherapy and radiation treatment may substantially impair performance status and QOL but that there are other important disease- and treatment-related factors to be considered as well.

Using a very detailed and specific evaluation of the side effects of radiation therapy, Trotti et al. (39) found statistically significant increases from pretreatment to 4 weeks into radiation therapy in the following areas: pain in throat, difficulty

swallowing breads/meats and liquids, changes in mucous and saliva, changes in taste, difficulty chewing, and speech difficulties. The only potential mucositis-related symptom not to increase over that 4-week period was pain in the mouth. In addition, there was a very strong correlation (.85) between the total score for these specific symptoms and a general QOL measure, highlighting their importance to overall well-being during treatment.

In another investigation (40), QOL was assessed repeatedly throughout a sequential, multimodal protocol for the treatment of advanced head and neck cancer. Patients received either chemotherapy + surgery + radiation therapy or chemotherapy + radiation therapy only. The interesting results demonstrated a decrease in QOL from baseline to the second cycle of chemotherapy for all patients, poorer QOL in patients following surgery, and the worst QOL for both groups of patients during radiation therapy. Although this is a report of only a small number of patients, it demonstrates the utility of multiple, longitudinal QOL assessment and comparison between treatment arms.

Several longitudinal studies have examined both short- and long-term QOL-related treatment effects. For example, both disease-specific QOL and general QOL were assessed before radiotherapy for laryngeal cancer, as well as 6 and 12 months after the therapy (41). There was a statistically significant increase in head and neck symptoms related to mucositis and fatigue and a decrease in physical functioning when comparing the baseline to the 6-month assessments. However, of interest is the finding that both emotional well-being and mood improved at the 6-month assessment. Thus, there is some discordance between the physical/functional and emotional domains of QOL at this time, when mucositis-associated symptoms appear to still be present. We note that this may suggest the importance of adaptive psychological processes: Better mood, even in the face of continuing symptoms, may also be a function of relief at the end of active cancer treatment.

Pretreatment factors and type of treatment can also be predictive of post-treatment QOL and symptoms (42). Thus, a high level of depression and a low performance status at baseline and the receipt of combination therapy predicted increased symptom severity following the end of treatment. More intensive treatment was associated with greater head and neck cancer-specific symptomatology, while depression and performance status were better predictors of overall QOL.

Finally, there has been at least one attempt to systematically examine the relative importance of different head and neck symptoms/treatment effects to global QOL (43). This type of work will be particularly important in comparing treatments that have different short- or long-term side effect profiles. Subjects had no evidence of disease and had completed treatment within the last 2–12 months. They filled out a survey examining speech, eating, aesthetics, pain/discomfort, social/role functioning, and global QOL. In univariate analyses, social/role functioning and speech had the strongest association with global QOL. In a logistic regression designed to examine the relative predictive ability of each of the domains, speech and eating were the best independent predictors of global QOL. Although this study included a relatively small number of subjects and, thus, clearly needs to be replicated in a large, representative sample, it does represent information that may be critically important to the evaluation of patient preferences for various treatment modalities.

Research on the specific economic and QOL impact of mucositis, independent of other head and neck treatment/disease-related problems, is rather limited. Summary statements about the likely negative impact of mucositis abound in the head and neck cancer literature. The extant empirical work reviewed above provides apparently compelling support for these claims but is all based on inference. That is, the relationship between mucositis severity, specifically, and QOL was not reported in any of the investigations. Rather, it was necessary to make the assumption either that mucositis is more severe in those receiving treatment or that patients' reports of symptoms, such as pain and swallowing difficulty, are related to mucositis. While a logical and useful first step, our understanding of the specific QOL impact of mucositis would clearly be enhanced by a prospective longitudinal evaluation of mucositis severity, symptoms, functional status, and global- and head and neck cancer-specific QOL. Such studies would also allow us to explore the potentially complex relationship between physician-graded toxicity (mucositis), patient-reported specific symptom severity, and multiple domains of QOL. It will be particularly interesting to examine the way in which mucositis and related symptomatology, such as eating and communication difficulties, affect social well-being.

Another important line of research will be that of evaluating patients' preferences for the potentially different acute and long-term consequences of increasingly aggressive treatment protocols. That is, careful explication of the QOL implications of different treatments (i.e., what it will really mean to a patient's sense of well-being in various areas) may inform treatment decision making, particularly in the absence of a clear survival advantage of one treatment versus another. List et al. (44) have demonstrated that, while cure and long-term survival are the most desired treatment outcomes, rankings of other outcomes, such as less pain and improved level of eating and communication ability, vary statistically significantly among patients. The provision of accurate information on short- and long-term treatment effects, such as mucositis and QOL, would help to address treatment-planning difficulty created by normal interpatient variability in preferences.

Finally, a careful prospective evaluation of the economic costs associated with the management of mucositis would be useful. The methodology utilized in our retrospective study reported above, with resource identification and costing strategies, is similar to that used in prospective studies of the costs related to other treatment side effects. Such a prospective economic evaluation will be particularly helpful given the increasing evaluation of the clinical utility of protective methods, such as granulocyte colony-stimulating factors (45), that are designed to minimize mucositis during intensive head and neck cancer treatment. Cost-effectiveness or cost-benefit analyses could be conducted with knowledge of the true costs of mucositis management and the costs and efficacy of such protective agents.

In summary, the rapid expansion of antineoplastic treatment regimens in head and neck cancer, the often dose-limiting toxicity of mucositis, the evaluation of new protective agents, and the substantial heterogeneity of patients' evaluation of symptoms and QOL make the continued evaluation of the QOL and economic impact of mucositis an important and exciting area of investigation.

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NOTE

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The May 25, 2000, plenary session highlighted discussions that emerged from each of the workgroups.

The workgroups themselves had been organized on the basis of the following scientific themes:

- 1) Mucosal Biology I: Mouth and Esophagus
- 2) Mucosal Biology II: Intestine
- 3) Mucosal Immunology
- 4) Infection and Mucosal Injury
- 5) Gene Expression and Inflammation
- 6) Biology of Mucosal Pain
- 7) Mucosal Drug Delivery
- 8) Quality of Life and Economics

The "Appendix" section lists the guidelines that were used for each of the workgroups. Conference participants attended a May 24, 2000, plenary session conducted before the workgroups, at which time invited speakers addressed these subjects.

A summary of plenary session outcomes follows.

COMMON WORKSHOP THEMES

Several themes common to all workgroups evolved during plenary discussion in relation to mucosal injury in cancer:

- 1) Mucositis in cancer patients is multifactorial in nature.
- 2) A balance exists at the clinical level between the need to treat cancer and the relationship of that treatment to causing mucosal injury.
- 3) It is important to interface appropriate patient-oriented outcomes with objective outcomes relative to clinical trial design and Federal regulatory mandates.
- 4) There is need for innovative *in vitro*, *in vivo*, and clinical models for mucosal injury. These models could include identification of surrogate markers; examination of relationships of oral to other gastrointestinal mucositis; interrelationships of pain models with those for mucositis; patient-related risk factors, including the potential role of genomics in risk for and cause of mucositis; mucositis scoring systems; mucositis as a model for wound healing; and economic models relative to acute and chronic complications associated with mucositis, with attention paid to the variety of health care models that exist internationally. Ultimately, the best model is the human model.
- 5) Considerable opportunities exist for academic, clinical, industrial, and government collaborations. It is critically important that standardized approaches be utilized, including mucositis nomenclature as well as scoring system techniques.
- 6) Effective communication and multiprofessional education are essential to advance the research agenda. Examples by which this can occur include publication of the enclosed conference proceedings; development of a listserve linked to a website directed to research and clinical issues regarding mucosal injury in cancer; and participation in the activities of professional organizations, including the Multinational Association of Supportive Care in Cancer, currently operating in alliance with the International Society for Oral Oncology.

In addition to these common themes, issues specific to a given workgroup were also addressed in the plenary session. A summary of these discussions follows.

Mucosal Biology I: Mouth and Esophagus— Dr. Christopher A. Squier, Chair

Because mucositis is a multifactorial disease, it is essential that endpoints be standardized. In addition to scientific advantages, this will reduce potential regulatory conflicts when multiple Federal and institutional review groups are involved.

Relative to laboratory models, there appears to be value in studying the unique contributions of connective tissue, epithelium, and oral microorganisms. *In vitro* models should compare oral epithelial injury in relation to intestinal mucositis, including the role of inflammatory infiltrates and the measurement of apoptotic changes in the two respective anatomic sites. The hamster model currently used for several mucositis studies has represented an important contribution to science. However, the model itself is only as effective as the questions being asked. Furthermore, it will be important to determine what relevant cellular and molecular components in the mucositis model are conserved across species.

Clinical models should address differences in mucositis signs and symptoms across all mucosal surfaces, correlation between morphologic changes and subjective feeling of pain, possible role of tobacco as a risk factor for mucositis, and long-term sequelae of mucosal damage.

Definitions for stomatitis are not uniformly applied in relation to mucositis caused by radiation therapy versus chemotherapy. This disparity is reflected in current measurement systems, including the National Cancer Institute's Common Toxicity Criteria.

Mucosal Biology II: Intestine—Dr. Christopher S. Potten, Chair

Recent research has been principally directed to causes and management of oral mucositis. Additional research studies are needed relative to mucositis occurring at other gastrointestinal sites. This research should include

- 1) Improved characterization of cell biology and innate mechanisms for injury and repair. Selected studies should specifically explore the possible influence of circadian rhythms associated with small and large intestine kinetics. These rhythms appear to differ between rodents and humans.

Affiliations of authors: D. E. Peterson, School of Dental Medicine, Department of Oral Diagnosis, University of Connecticut Health Center, Farmington; S. T. Sonis, Brigham and Women's Hospital and Dana-Farber Cancer Institute, Divisions of Oral Medicine, Oral and Maxillofacial Surgery, and Dentistry, Boston, MA.

Correspondence to: Douglas E. Peterson, D.M.D., Ph.D., School of Dental Medicine, Department of Oral Diagnosis, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1605 (e-mail: Peterson@NSO.UCHC.EDU).

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- 2) Systemic and topical drug delivery systems to nonoral alimentary tract sites. These systems could be coupled by agents that preserve mucosal barrier function through protecting cells in replicative phase and by activating cellular repair processes. This could occur via incorporation of DNA repair genes into intestinal stem cells and via tissue engineering, modeled after research involving skin grafts for burn patients.
- 3) Development of novel diagnostic approaches, to supplement currently used indirect measures including pain and diarrhea.

Mucosal Immunology—Dr. Leo Lefrancois, Chair

Additional research is needed regarding:

- 1) The role of the immune system relative to damage and repair during the multiple phases of oral mucositis.
- 2) The hierarchy of loss and the recovery of immune components during mucositis. Examples of these phenomena include loss of lymphocytes, antigen-processing cells, and polymorphonuclear leukocytes, followed by their subsequent resurgence, and the relationship of peripheral blood-immune components to tissue-based elements.
- 3) The delineation of cellular and molecular factors potentially responsible for induction and repair of tissue damage, including cytokines (e.g., those principally responsible for modulation of epithelial cell kinetics) and the relationship to destruction of stem cells.
- 4) The mechanisms governing tissue-specific effects, to better understand why selected mucosal sites are commonly affected while other sites are not. Application of current models of inflammatory bowel disease may be useful in this regard.

Chemotherapy regimens for humans do not translate well to study in nonhuman primates and other animals. Improved animal models for oral mucositis and gut diseases are thus needed. The rat may be particularly relevant to this research.

Infection and Mucosal Injury—Dr. John R. Wingard, Chair

Basic research should address both transgenic knockout mice as an important technology to examine mucosal stem cell biology and mechanisms that render oral mucosa more vulnerable to injury than other, nonoral, mucosal tissue. Differences could be related to cell cycling, microbial flora, degree of physical trauma, kinetics of drug delivery, and/or salivary contributions in the oral cavity.

Several agents exist relative to mucositis prevention or treatment. It is important to study the most promising interventions via randomized clinical trials.

Critical attention must be paid to the definition and selection of endpoints in these clinical trials, including the strength of the data justifying use of the specific endpoint, the feasibility of measurements, and the clinical significance of differences generated via different mucosal scoring systems.

An important goal is to develop precision in endpoint design and implementation consistent with clinical trials in noncancer cohorts. The hypertension model appears to be an effective example of such precision.

Mucositis is clearly related to increased risk for systemic infection in the neutropenic cancer patients. In addition to research relative to mucosal biology and disease, further study of

locally injurious mechanisms directly mediated via oral bacteria or fungi is important.

Mucositis in cancer patients can compromise delivery of cancer therapy. This is a serious risk, since maintenance of cancer therapy dose intensity is important for maximizing patient survival.

Gene Expression and Inflammation—Dr. David A. Williams, Chair

In vitro systems, including clonogenic assays, are at present relatively limited in sophistication. Strategic new technology development in this arena is required.

Important research issues to be pursued include defining the role of genetics and the potential that genes amplify and thus alter susceptibility to mucositis; the mechanisms by which endothelial damage may lag behind that observed in epithelium; the role of the stem cell in oral mucosal health and disease; the relationship between systemic myeloablation and mucosal disease; potential therapeutics, with attention paid to technologic and biologic barriers; and relationships between chemotherapy-induced mucositis and disease processes in other cancer and noncancer patients.

Biology of Mucosal Pain—Dr. Christine Miaskowski, Chair

Key research issues relating to both laboratory and clinical studies include identification of fundamental mechanisms of pain causation throughout the alimentary tract, the immunopathogenic basis of pain, potential biomarkers for pain measurement, the means by which patients discriminate types of pain and pain intensity, and relationships between acute and chronic pain experiences and their sequelae.

Improvement in animal systems for study of pain is needed, including

- 1) The extension of current animal models for pain to new models for pain associated with mucosal injury. For example, current technology in which phantom limb pain following amputation is reduced by prophylactic analgesics may have relevance to mucositis pain studies.
- 2) The role of genetic variability relative to tissue response to injury.

Clinical research needs to address

- 1) The relationship of risk factors to early identification of patients likely to experience significant pain secondary to mucositis.
- 2) The subjective assessments of pain, including incorporation of changes in patient perception as cancer therapy continues. For example, a patient may report a high pain score early in the treatment process. Subsequently, the patient's report may exceed the scale parameters if more severe pain develops.
- 3) The appropriate measures of the effect of pain in relation to economics of healthcare in cancer patients.
- 4) The improvement in strategies to collect and interpret data regarding the influence of pain medication on pain reporting in clinical trials.
- 5) The efficacy of topical versus systemic application of pain medication, including patient education relative to goals of pain treatment and importance of compliance with medication dosing.

- 6) New delivery systems for pain medication, including the potential utility of a microchip-based dermal or mucosal patch system.

Mucosal Drug Delivery—Dr. Vincent H. L. Lee, Chair

The potential full value of a mucoprotective drug may be realized by successfully tailoring its delivery to mucosa. Important research issues necessary to achieve this goal include

- 1) The development of new *in vitro* models to better define pathogenesis of mucositis in relation to strategies for drug formulation and dosing frequency.
- 2) The enhancement of drug delivery to mucosal surfaces in order to maximize drug efficacy, including drug distribution within tissue and increased patient tolerability via improvements in formulation.

A variety of scientific expertise is needed to effectively develop new drug delivery systems. Scientists, including geneticists, biomaterial scientists, bioinformatics specialists, and tissue engineers, should participate in this research in partnership with the appropriate health professionals.

Understanding the full scope of the mechanism by which cancer chemotherapeutic drugs are delivered to mucosal surfaces may permit identification of new approaches to reduce mucosal injury in patients.

Quality of Life and Economics—Dr. David Cella, Chair

Future research is needed in the following areas:

- 1) Efficacy of treatments to improve quality of life (QOL) in cancer patients.
- 2) QOL assessment and management in pediatric cancer populations.
- 3) Effect of mucositis in relation to long-term cancer patient outcomes, including survival.
- 4) Relationships of global versus subcategory measures of QOL to the acute effects of mucositis.
- 5) Potentially different responses to pain and analgesic medications based on sex. Although there is no apparent variation in susceptibility to mucositis between males and females, level of pain and response to therapy may differ between the sexes.
- 6) Assessment and management of gastrointestinal toxicity at anatomic sites other than the oral cavity.
- 7) Correlation of patient assessment of QOL with objective measures to define clinically important changes.
- 8) QOL associated with mucositis across different cancer populations. In addition, study of these issues in relation to QOL in noncancer cohorts including those with chronic pain, gut diseases with an inflammatory component, or chronic fatigue syndrome may be valuable.

New cost models are needed relative to management of oral mucositis in cancer patients. In addition, potential cost savings

associated with an institutional oral care program should be examined. These QOL and economic studies need to address multiple variables, including underlying disease diagnosis, patient demographics, and type of cancer treatment.

Development of a multi-institutional registry for oral complications in cancer, including mucositis, may be useful at both scientific and clinical levels.

APPENDIX

GUIDELINES FOR WORKGROUP DISCUSSION (DISTRIBUTED IN ADVANCE TO CONFERENCE PARTICIPANTS)

Conference objective. To strategically advance basic, translational, and applied knowledge needed to ultimately prevent or ameliorate significant mucosal injury associated with intense cytotoxic cancer therapy.

Workgroup objective. To generate specific basic, translational, and applied research ideas involving the workgroup's topic in relation to mucosal toxicity.

Discussion points for each workgroup. (Please note: dialogue should be based on the collective experience of the group as well as the reviews presented in the May 24 plenary session. The report from each workgroup should follow the format below. Additional questions and discussion can be reported in Section 6. The report will be presented by the workgroup chair at the May 25 plenary session. Each presentation will consist of a 10-minute summary followed by an open discussion.)

- 1) What is the strength of data relative to the following factors as studied in laboratory or clinical models for mucosal injury in cancer?
 - Degree of risk (low, moderate, or high)
 - Cause
 - Diagnosis
 - Prevention and treatment
 - Clinically significant local or systemic sequelae
 - Impact on quality of life and health care costs
 - Relationship to cancer cure or remission
- 2) What models of mucosal injury exist in noncancer settings that could define new strategies for research in relation to mucosal injury in cancer?
- 3) What key research questions could substantially advance knowledge of mucosal injury in cancer?
- 4) What is the degree of relevance of the research questions (Section 3) to important clinical outcomes?
- 5) In what ways could current and proposed research regarding mucosal injury in cancer enhance understanding of mucosal injury in noncancer models?
- 6) What additional questions and discussion did the workgroup address?

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*The National Institutes of Health Consensus
Development Conference: Adjuvant Therapy for
Breast Cancer*

2001
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Adjuvant Therapy for Breast Cancer**

Bethesda, Maryland

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Monograph Overview

Jeffrey S. Abrams, Patricia Eifel

Beginning in 1977, the National Institutes of Health (NIH), the lead governmental agency for federally sponsored research in the United States, organized Consensus Development Conferences to review controversial areas of medical research (1). More than 120 such conferences have been held on a wide variety of diseases and medical disciplines. Adjuvant therapy of breast cancer has been one of the most popular topics for Consensus Development Conferences, with previous meetings held in 1980, 1985, and 1990 (2–4). As 10 years had elapsed since the last conference, the National Cancer Institute at NIH thought that it was time to once again cosponsor a Consensus Development Conference to review what progress had occurred in adjuvant breast cancer therapy.

Consensus Development Conferences are structured to emphasize a balanced, critical review of the scientific evidence. A planning committee develops several key questions of paramount interest. It then selects experts in the field to present pertinent research results to an independent, nongovernmental panel consisting of academic and community-based physicians, biostatisticians, nurses, basic researchers, and other appropriate disciplines, along with lay patient advocates. The panelists are selected for their general expertise in their chosen field, but they cannot have published extensively or be considered “opinion leaders” on the topic selected for consensus development.

Held November 1–3, 2000, the Consensus Conference on Adjuvant Therapy of Breast Cancer was open to the public, and admission was free. Discussion sections were interspersed throughout the formal presentations to provide both panelists and members of the audience an opportunity to query the speakers or to offer additional evidence. At the conclusion of the expert presentations, the panel met in a closed session and developed a draft Consensus Statement. The panel chairperson read the entire draft to all of the meeting participants, who offered comments and suggestions that were carefully considered by the panelists before they finalized the statement. At the close of the conference, the Consensus Statement was presented to the national press and made available on the NIH Web site (<http://consensus.nih.gov>) and has subsequently been published (5). The entire statement is reproduced in this monograph. The statement is intended to provide useful summary recommendations to professionals and the public that reflect the current state of the art.

The planning committee believed that a broad review of adjuvant therapy was required to help clinicians deal with practical problems in selecting the appropriate adjuvant therapy. The following six questions were posed to the panel: 1) Which factors should be used to select systemic adjuvant therapy? 2) For which patients should adjuvant hormonal therapy be recommended? 3) For which patients should adjuvant chemotherapy be recommended, and which agents should be used and at what dose or schedule? 4) For which patients should postmastectomy radiotherapy be recommended? 5) How do side effects and quality-of-life issues factor into individual decision making about adju-

vant therapy? 6) What are promising new research directions for adjuvant therapy?

Before the conference, the panelists reviewed the existing medical evidence from the literature. Their review was then supplemented at the meeting by presentations from 33 expert speakers. This monograph presents the details of the evidence offered by the experts on which the consensus statement was largely based. Of the 33 presentations, all but nine speakers have contributed original articles to this monograph. In the overview of this monograph that follows, we mention the reviews by the contributing authors but focus our attention primarily on the presentations not included in the monograph, so the reader has a complete review of the basis for the consensus recommendations. The full name of the presenter and his or her affiliation is provided for those who did not submit articles, while the reader can find this information for those who contributed in the original articles that follow.

QUESTION 1: WHICH FACTORS SHOULD BE USED TO SELECT SYSTEMIC ADJUVANT THERAPY?

Progress in the development of new prognostic and predictive factors for adjuvant therapy has been difficult to realize. Traditional tumor-related factors, such as axillary lymph node status, tumor size, histologic type and grade, and hormone receptor status, were still the most useful indicators of prognosis, while receptor status was the most valuable predictor of response to endocrine therapy (6). These issues are reviewed thoroughly by Drs. Gary Clark, Stuart Schnitt, and Maria Grazia Daidone. Their presentations highlighted the fact that all too often, markers are touted before definitive studies are available to prove their value independent of established factors. In some cases, even when new markers have been shown to be independent predictors, the assays were either not reliable or were not generally available. Although poised technologically to make important advances in coming years, research in this area is dependent on the ability to amass large sample sizes with careful documentation of the patient, tumor, and treatment characteristics of the study population. Phase III cooperative group treatment trials in this country and abroad increasingly have such features and are potentially valuable sources of specimens. However, carefully performed clinical trials were thought to be only an initial step. New markers developed in the research laboratory must be transferable to general medical care before they can make an impact on clinical decision making.

Drs. James Dignam, Aron Goldhirsch, and Hyman Muss examined patient-specific characteristics including racial/ethnic

Affiliations of authors: J. S. Abrams, Clinical Investigations Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Rockville, MD; P. Eifel, Department of Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, Houston.

Correspondence to: Jeffrey S. Abrams, M.D., National Institutes of Health, 6130 Executive Blvd., EPN 7040, Rockville, MD 20852 (e-mail: Abramsj@ctep.nci.nih.gov).

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background, young age, and older age, respectively. Although breast cancer is a common disease in older women, relatively few older women participated in clinical trials, so their treatment was often extrapolated from younger populations. This problem was in need of remediation by the research community. For younger women, data now suggest that hormonal therapies are extremely important for hormone-sensitive tumors. As for race or ethnicity, a differential treatment effect was not detected according to these factors.

QUESTION 2: FOR WHICH PATIENTS SHOULD ADJUVANT HORMONAL THERAPY BE RECOMMENDED?

Multiple clinical trials supported the panel's recommendation that tamoxifen should be considered in all women with hormone receptor-positive breast cancer, irrespective of age, menopausal and lymph node status, tumor size, and treatment with chemotherapy (7). The only exception cited was for women with very small tumors, less than or equal to 1 cm, for whom the side effects might outweigh the advantages. Sir Richard Peto (Oxford University, England) presented the evidence supporting this recommendation by reviewing updated results from the September 2000 meta-analysis performed by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Comparing women treated with and without tamoxifen for 5 years, the results in 8000 women with estrogen receptor-positive tumors indicated that tamoxifen reduced death from breast cancer by $9\% \pm 1.4\%$ at 15 years without increasing non-breast cancer deaths statistically significantly. The sole prognostic factor in the meta-analysis that correlated with survival was hormone receptor status. Only women with positive estrogen receptors benefited from tamoxifen. Dr. Kent Osborne (Baylor University, Houston, TX) discussed whether tamoxifen was indicated for hormone receptor-negative disease. Neither the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-23 trial (8) nor the Inter-group trial (9) demonstrated an improvement in overall survival or a reduction in contralateral breast cancer in hormone receptor-negative tumors. This was also the finding of the EBCTCG September 2000 meta-analysis. On the basis of these facts, the panelists recommended that tamoxifen not be used in women with hormone receptor-negative disease.

Dr. Christina Davies (Oxford University, England) and Dr. Bryant debated the optimal duration of tamoxifen therapy. Dr. Davies presented the results of the September 2000 EBCTCG meta-analysis, which demonstrated that 5 years of tamoxifen compared with 1–2 years of tamoxifen produced an additional absolute survival increment of $3.2\% \pm 1\%$ at 10 years while causing only a 0.1% increase in such serious side effects as pulmonary embolus or endometrial cancer. The panelists concluded that 5 years of therapy should be recommended routinely, since the advantages of the drug outweighed its toxicity for most women with invasive breast cancer. Studies comparing 5 years with longer durations of therapy (7–10 years) did not reveal a benefit for therapy beyond 5 years. Dr. Davies maintained that these results are premature, arguing that the survival benefit from 5 years of tamoxifen, rather than remaining constant, continues to increase during the next 5 years ("carryover effect"), despite the drug being stopped. An advantage for prolonged therapy, she reasoned, might be expected to emerge only after 10–15 years of follow-up. In support of this contention, more recurrences have occurred in the placebo ($n = 16$) than in the tamoxifen ($n = 11$) group in B-14 when only recurrences diag-

nosed more than 6 years after randomization were examined (10). However, Dr. Bryant contended in his article that the number of events during this time period is quite small, while the overall study results from B-14 continued to indicate no statistically significant survival advantage for the longer therapy, a finding similar to analyses from the Scottish (11) and Eastern Cooperative Oncology Group (12) trials of tamoxifen duration. Outside of clinical trials, the panelists recommended limiting tamoxifen treatment to 5 years.

Dr. Nancy Davidson reviewed ovarian ablation, an alternative to tamoxifen. The evidence that she described convinced the panelists that ovarian ablation is an acceptable adjuvant therapy in women with premenopausal, hormone-responsive breast cancer. The panelists recommended that further clinical trials were needed to determine whether ablation was additive when combined with tamoxifen and chemotherapy.

QUESTION 3: FOR WHICH PATIENTS SHOULD ADJUVANT CHEMOTHERAPY BE RECOMMENDED, AND WHICH AGENTS SHOULD BE USED AND AT WHAT DOSE OR SCHEDULE?

Chemotherapy remained the systemic adjuvant therapy of choice for most patients with hormone receptor-negative breast cancer. Dr. Gabriel Hortobagyi presented a thorough review of progress in the field over the past decade. As demonstrated in earlier EBCTCG overviews (13) and at the most recent update in September 2000, all patient subsets, lymph node-negative/positive, pre/postmenopausal, and hormone receptor-negative/positive, derived a statistically significant survival advantage, in the range of 3%–12%, from chemotherapy compared with no chemotherapy. As anticipated, the absolute benefit was greatest for women at highest risk of relapse. Regarding specific regimens, the September 2000 overview also indicated that anthracycline-containing regimens yield a 3.5% absolute improvement in survival.

For commonly used drugs such as doxorubicin and cyclophosphamide, clinical trials have demonstrated a threshold dose below which these drugs appear less effective, but dose escalation beyond the threshold level has not been demonstrated to lead to increased benefit. Dr. Larry Norton (Memorial Sloan-Kettering Cancer Institute, New York, NY) discussed several clinical trials that tested dose escalation without the need for stem cell support. He concluded that the optimal doses of cyclophosphamide and doxorubicin were 600 mg/m^2 and 60 mg/m^2 , respectively, for adjuvant breast cancer treatment. A key unanswered question, he noted, was whether combination or sequential use of active agents is most beneficial.

Dose escalation was also reviewed in the context of high-dose chemotherapy trials with autologous stem cell support. Dr. William Peters (Barbara Ann Karmanos Cancer Institute, Detroit, MI) presented an update of Cancer and Leukemia Group B (CALGB) 9082 (14). He found no difference in disease-free or overall survival with a median follow-up of 5.1 years between the high-dose arm with autologous stem cell support and an intermediate-dose arm using only granulocyte-colony stimulating factor support. Dr. Karen Antman reviewed results from five additional international randomized trials using high-dose therapy. Although a Dutch trial has encouraging preliminary results (15), the other trials did not indicate a survival advantage. Nine additional international trials were either ongoing or had

completed accrual but are not yet analyzed. Until and unless additional clinical trials became available that demonstrate a benefit for high-dose therapy, the panelists concluded that this approach should not be used in routine practice.

Dr. Norman Wolmark discussed neoadjuvant breast cancer therapy, noting that this approach had the potential to markedly shorten the time it takes to evaluate promising agents. Dr. Richard Gray (University of Birmingham, England) presented results from the September 2000 EBCTCG meta-analysis regarding comparisons of chemotherapy and hormonal therapy. He noted that, for women 50 years of age or more with hormone-sensitive tumors, tamoxifen yielded a greater survival benefit than did chemotherapy. However, the treatments generally appeared additive, with the combination producing superior survival results than either modality alone in most tumor subsets analyzed. The panel concluded that tamoxifen should be used whenever chemotherapy is considered for receptor-positive tumors.

The most controversial issue faced by the panelists concerned the role of taxanes as part of adjuvant chemotherapy regimens. Dr. Craig Henderson (University of California, San Francisco) updated CALGB 9082, a U.S. Intergroup study (16). The study tested doxorubicin and cyclophosphamide (AC) for four cycles followed by paclitaxel for four cycles versus AC alone. With a median follow-up of 52 months, a statistically significant disease-free and overall survival advantage for the addition of paclitaxel persists. An unplanned subset analysis indicates that the advantage is limited to patients who did not receive tamoxifen. Dr. Eleftherios Mamounas (Aultman Cancer Center, Canton, OH) presented an interim analysis of NSABP B-28. This study was similar in design to the CALGB trial. With a median follow-up of 34 months, no disease-free or overall survival advantage was noted for the addition of paclitaxel to AC chemotherapy. A subset analysis has not indicated a statistically significant difference according to whether or not tamoxifen was administered. However, similar to the NSABP trial, a trend favoring paclitaxel was noted in patients who did not receive tamoxifen or who had hormone receptor-negative tumors. Dr. Martine Piccart discussed important differences between these two trials that might explain their results. Further follow-up of the NSABP trial and completion of other ongoing randomized trials examining the role of taxanes should clarify whether these agents will become a part of standard chemotherapy regimens. The panel concluded that definitive evidence was lacking regarding the role of taxanes but counseled against their usage in patients with lymph node-negative breast cancer outside clinical trials.

To determine whether subsets of patients exist for whom the risk of recurrence does not justify the routine use of chemotherapy, Drs. Monica Morrow and Jonas Bergh analyzed population-based registry data from the United States and Europe, respectively. Their reviews suggested excellent outcomes without adjuvant therapy in selected subsets of lymph node-negative tumors with small tumors. In contrast to the registry data, Dr. Bernard Fisher compiled results across several NSABP lymph node-negative, randomized clinical trials that compared various systemic therapies. Selected subsets of women in these trials seemed to benefit from adjuvant hormonal therapy and/or chemotherapy. In view of the small yet statistically significant benefits for some patients with tumors considered to be at low risk, the challenge for physicians is to provide a balanced explanation of risks and benefits that will permit individuals to make an informed, personal decision.

QUESTION 4: FOR WHICH PATIENTS SHOULD POSTMASTECTOMY RADIOTHERAPY BE CONSIDERED?

Dr. Jack Cuzick (Imperial Cancer Research Fund, London, England) updated the recent EBCTCG meta-analysis (17) that demonstrated that adjuvant radiotherapy produces an absolute reduction of 20% in isolated local recurrences at 20 years, irrespective of the use of chemotherapy or tamoxifen. This large reduction in local recurrence was accompanied by a statistically significant absolute reduction in death by breast cancer of 5% at 15 years. However, a concomitant absolute increase of 4% in non-breast cancer deaths occurred in patients receiving radiotherapy. This increase did not begin to appear until about 5 years after treatment and seemed to increase at least out to 15 years after treatment. A stratified analysis, performed according to whether trials were initiated before or after 1975, indicated that the trials started before 1975 had a very statistically significant increase in non-breast cancer deaths, while those started after 1975 did not. This might be because of the use of improved radiotherapy techniques in the latter trials, but longer follow-up of the trials in this latter group was needed before this explanation could be fully accepted. It was also noteworthy that the majority of non-breast cancer deaths, although vascular in origin, were not cardiac related.

To assess who should receive postmastectomy radiotherapy, Dr. Lori Pierce reviewed several risk factors potentially associated with local recurrence. On the basis of the information presented, the panelists agreed that postmastectomy radiotherapy was indicated for women with four or more positive axillary lymph nodes and for those with large primary tumors. The benefit was less clear for those with one to three positive lymph nodes, and participation of such patients in an ongoing Southwest Oncology Group clinical trial (S9927) testing this question was recommended.

QUESTION 5: HOW DO SIDE EFFECTS AND QUALITY-OF-LIFE ISSUES FACTOR INTO INDIVIDUAL DECISION MAKING ABOUT ADJUVANT THERAPY?

Ms. Amy Langer introduced the subject by explaining how patients view these issues and how they affect decision making. Drs. Patricia Ganz and Eric Winer described the side effects of tamoxifen and chemotherapy, respectively. The value of adjuvant therapy, a mix of benefits counterbalanced by risks, requires physicians to present detailed information to patients. Dr. Mark Levine reviewed research on how best to present information to patients. However, once the information is understood, the ultimate decision remains a personal one based on the individual's tolerance of risk and the value attributed to the benefits. Dr. Alan Coates described several studies that assessed the willingness of patients to receive adjuvant therapy based on scenarios that present differing degrees of survival gains.

The panelists concluded that effective communication between physicians and patients is essential to the decision-making process regarding adjuvant therapy. Complex information must be conveyed regarding absolute risks and benefits, so patients can appreciate the trade-offs involved in their choices. This process can be facilitated through the use of decision aids and other well-designed patient information materials.

QUESTION 6: WHAT ARE PROMISING NEW RESEARCH DIRECTIONS FOR ADJUVANT THERAPY?

Integrating genomics and proteomics into the search for prognostic factors, testing the optimal duration and combinations of hormonal treatments and the value of chemotherapy in older patients, and including quality-of-life endpoints in adjuvant trials were all cited during the conference as important future research directions. The primary new therapeutic direction involved targeted therapies based on an improved understanding of molecular pathways. Herceptin and bisphosphonates were among the first of this new generation of targeted treatments to enter adjuvant clinical trials. Many other agents that target specific proliferative pathways are being studied in patients with advanced breast cancer. The challenge for the future is to integrate the most promising of these pathways into the current adjuvant therapy armamentarium.

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National Institutes of Health Consensus Development Conference Statement: Adjuvant Therapy for Breast Cancer, November 1–3, 2000

*National Institutes of Health Consensus Development Panel**

Objective: Our goal was to provide health-care providers, patients, and the general public with an assessment of currently available data regarding the use of adjuvant therapy for breast cancer. **Participants:** The participants included a non-Federal, non-advocate, 14-member panel representing the fields of oncology, radiology, surgery, pathology, statistics, public health, and health policy as well as patient representatives. In addition, 30 experts in medical oncology, radiation oncology, biostatistics, epidemiology, surgical oncology, and clinical trials presented data to the panel and to a conference audience of 1000. **Evidence:** The literature was searched with the use of MEDLINE® for January 1995 through July 2000, and an extensive bibliography of 2230 references was provided to the panel. Experts prepared abstracts for their conference presentations with relevant citations from the literature. Evidence from randomized clinical trials and evidence from prospective studies were given precedence over clinical anecdotal experience. **Consensus Process:** The panel, answering predefined questions, developed its conclusions based on the evidence presented in open forum and the scientific literature. The panel composed a draft statement, which was read in its entirety and circulated to the experts and the audience for comment. Thereafter, the panel resolved conflicting recommendations and released a revised statement at the end of the conference. The panel finalized the revisions within a few weeks after the conference. The draft statement was made available on the World Wide Web immediately after its release at the conference and was updated with the panel's final revisions. The statement is available at <http://consensus.nih.gov>. **Conclusions:** The panel concludes that decisions regarding adjuvant hormonal therapy should be based on the presence of hormone receptor protein in tumor tissues. Adjuvant hormonal therapy should be offered only to women whose tumors express hormone receptor protein. Because adjuvant polychemotherapy improves survival, it should be recommended to the majority of women with localized breast cancer regardless of lymph node, menopausal, or hormone receptor status. The inclusion of anthracyclines in adjuvant chemotherapy regimens produces a small but statistically significant improvement in survival over non-anthracycline-containing regimens. Available data are currently inconclusive regarding the use of taxanes in adjuvant treatment of lymph node-positive breast cancer. The use of adjuvant dose-intensive chemotherapy regimens in high-risk breast cancer and of taxanes in lymph node-negative breast cancer should be restricted to randomized trials. Ongoing studies evaluating these treatment strategies should be supported to determine if such strategies have a role in adjuvant treatment. Studies to date have included few patients older than 70 years. There is a critical need for trials to evaluate the role of adjuvant

chemotherapy in these women. There is evidence that women with a high risk of locoregional tumor recurrence after mastectomy benefit from postoperative radiotherapy. This high-risk group includes women with four or more positive lymph nodes or an advanced primary cancer. Currently, the role of postmastectomy radiotherapy for patients with one to three positive lymph nodes remains uncertain and should be tested in a randomized controlled trial. Individual patients differ in the importance they place on the risks and benefits of adjuvant treatments. Quality of life needs to be evaluated in selected randomized clinical trials to examine the impact of the major acute and long-term side effects of adjuvant treatments, particularly premature menopause, weight gain, mild memory loss, and fatigue. Methods to support shared decision-making between patients and their physicians have been successful in trials; they need to be tailored for diverse populations and should be tested for broader dissemination. [J Natl Cancer Inst Monogr 2001;30:5–15]

Each year, more than 180 000 women in the United States are diagnosed with breast cancer, the most common type of noncutaneous cancer among women in this country. If current breast cancer rates remain constant, a woman born today has a one in 10 chance of developing breast cancer.

Because of continuing research into new treatment methods, women with breast cancer now have more treatment options and a better chance of long-term survival than ever before. The primary treatment of localized breast cancer is either breast-conserving surgery and radiation therapy or mastectomy with or without breast reconstruction. Systemic adjuvant therapies that are designed to eradicate microscopic deposits of cancer cells that may have spread or metastasized from the primary breast cancer have been demonstrated to increase a woman's chance of long-term survival.

Systemic adjuvant therapies include chemotherapy (anticancer drugs) and hormone therapy. In addition to these systemic therapies, radiotherapy is used in selected cases as a local adjuvant treatment to destroy breast cancer cells that remain in the chest wall or regional lymph nodes after mastectomy.

The rapid pace of discovery in this area continues to expand the knowledge base from which informed treatment decisions can be made. The purpose of this conference was to establish a

*The members of the Consensus Development Panel are the authors of this article (see page 10).

Correspondence to: John A. Bowersox, Communications Specialist, Office of Medical Applications of Research, National Institutes of Health, Bldg. 31, Rm. 1B03, Bethesda, MD 20892 (e-mail: bowersoj@od.nih.gov).

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consensus regarding the use of adjuvant therapy for breast cancer and to communicate that consensus to clinicians, patients, and the general public. After reading relevant literature and attending a day and a half of presentations and audience discussion, an independent, non-Federal consensus development panel weighed the scientific evidence and drafted a statement that was presented to the conference audience on the third day. The Consensus Development Panel's statement addresses the following key questions:

- 1) Which factors should be used to select systemic adjuvant therapy?
- 2) For which patients should adjuvant hormonal therapy be recommended?
- 3) For which patients should adjuvant chemotherapy be recommended? Which agents should be used and at what dose or schedule?
- 4) For which patients should postmastectomy radiotherapy be recommended?
- 5) How do side effects and quality-of-life issues factor into individual decision-making about adjuvant therapy?
- 6) What are promising new research directions for adjuvant therapy?

1) WHICH FACTORS SHOULD BE USED TO SELECT SYSTEMIC ADJUVANT THERAPY?

The selection of systemic adjuvant therapy is based on prognostic and predictive factors. Prognostic factors are measurements available at diagnosis or at the time of surgery that, in the absence of adjuvant therapy, are associated with recurrence rate, death rate, or other clinical outcome. Predictive factors are measurements associated with the degree of response to a specific therapy. For example, a demonstration of hormone receptors in tumor cells predicts the response to hormonal therapy. Any factor has the potential to be both prognostic and predictive, and a factor's importance depends on both the clinical endpoint and the method of treatment comparison.

Prognostic and predictive factors fall into three categories: 1) patient characteristics that are independent of the disease, such as age; 2) disease characteristics, such as tumor size and histologic type; and 3) biomarkers (measurable parameters in tissues, cells, or fluids), such as estrogen receptor status, progesterone receptor status, and measures of cell turnover. Accepted prognostic and predictive factors include age, tumor size, axillary lymph node status, histologic tumor type, standardized pathologic grade, and hormone receptor status.

The median age for the diagnosis of breast cancer in women is between 60 and 65 years. Some younger women (particularly <35 years old) have a more aggressive form of the disease, characterized by larger tumors of higher grade with vascular invasion. Elderly women (>70 years old) with breast cancer frequently have hormone receptor protein in their malignant tissue, suggestive of a more indolent tumor pattern and a high likelihood of response to hormonal therapy.

Race appears to be a prognostic but not a predictive factor. In contrast to white women with breast cancer, black women with breast cancer are generally younger and often have larger tumors at diagnosis, and a smaller percentage of black patients have hormone receptors in their tumor tissue. These factors contribute to a poorer prognosis. In cases of similar clinical presentation, however, adjuvant treatment confers similar benefits to both

black and white women. Research is needed on the benefits and risks of adjuvant therapy in Hispanic, Asian, and Native American women.

Novel technologies, such as tissue and expression microarrays and proteomics, present exciting potential, but their integration into clinical practice will depend on the proper design and analysis of clinical investigations. The same is true for measures of HER-2/neu overexpression or p53 status, histologic evidence of vascular invasion, and quantitative parameters of angiogenesis. These factors have been extensively studied both clinically and biologically, but they do not have an established role in patient management. For example, although overexpression/amplification of HER-2/neu is associated with an adverse outcome in lymph node-positive patients and may predict the response to therapy, laboratory methods and the reporting of results require standardization before its predictive performance can be established.

The development of immunohistochemical and molecular methods to identify occult cancer cells (i.e., micrometastases) in histologically tumor-free axillary lymph nodes or bone marrow has raised questions as to whether such findings should alter the clinical stage and become a further indication for systemic adjuvant therapy. At present, the clinical significance of these findings remains uncertain, and they require assessment in prospective clinical trials before they directly affect patient management.

It is essential that the value of predictive and prognostic factors be evaluated in well-designed clinical studies that are based on standardized protocols and have sufficient statistical power. Because these standards are infrequently met, very few new prognostic or predictive factors have been validated in the last 10 years, and future progress will depend on greater attention to these standards. Promising pilot studies should be followed by a validation phase, during which alternative assays for the biomarker are evaluated in a head-to-head comparison and prognostic/predictive value is studied. Since no single study will have sufficient power to properly evaluate predictive value, results from these trials should be combined.

2) FOR WHICH PATIENTS SHOULD ADJUVANT HORMONAL THERAPY BE RECOMMENDED?

The decision on whether to recommend adjuvant hormonal therapy should be based on the presence of hormone receptors, as assessed by immunohistochemical staining of breast cancer tissue. If the available tissue is insufficient to determine hormone receptor status, it should be considered to be positive, particularly in postmenopausal women. The small subset of women whose tumors lack estrogen receptor protein but contain progesterone receptor also appears to benefit from hormonal therapy. The presence or absence of HER-2/neu overexpression should not influence the decision to recommend hormonal therapy.

The goal of hormonal therapy is to prevent breast cancer cells from receiving stimulation from estrogen. Such stimulation occurs primarily in tumors that contain hormone receptor protein. Estrogen deprivation can be achieved by (a) blocking the receptor through the use of drugs, such as tamoxifen; (b) suppression of estrogen synthesis through the administration of aromatase inhibitors (e.g., anastrozole) in postmenopausal women or luteinizing hormone-releasing hormone agonists (e.g., goserelin) in premenopausal women; or (c) destruction of the ovaries through surgery or external beam radiation therapy. The administration of cytotoxic chemotherapy may indirectly accomplish

this same effect by damaging estrogen-producing cells in the ovaries.

Adjuvant hormonal therapy should be recommended to women whose breast tumors contain hormone receptor protein, regardless of age, menopausal status, involvement of axillary lymph nodes, or tumor size. While the likelihood of benefit is related to the amount of hormone receptor protein in tumor cells, patients with any expression of hormone receptor in their tumor cells may still benefit from hormonal therapy. Such treatment has led to substantial reductions in the likelihood of tumor recurrence, second primary breast cancer, and death persisting for at least 15 years of follow-up. Possible exceptions to this recommendation include premenopausal women with tumors smaller than 10 mm who wish to avoid the symptoms of estrogen deprivation or elderly women with similarly sized cancers who have a history of venous thromboembolic episodes.

Tamoxifen is the most commonly used form of hormonal therapy. Randomized trials and a meta-analysis have shown that 5 years of tamoxifen are superior to 1–2 years of such treatment. Currently, there are no convincing data that justify the use of tamoxifen for longer than 5 years outside the setting of a clinical trial. Although tamoxifen has been associated with a slight but definite increased risk of endometrial cancer and venous thromboembolism, the benefit of tamoxifen treatment far outweighs its risks in the majority of women. Neither transvaginal ultrasonography nor endometrial biopsies are indicated as screening maneuvers for endometrial cancer in asymptomatic women taking tamoxifen. Tamoxifen may be combined with combination chemotherapy, particularly in premenopausal women; such combinations may further reduce the risk of recurrence. At this time, there are no data to support the use of raloxifene or aromatase inhibitors as adjuvant hormonal therapy.

For hormone receptor-positive premenopausal patients, alternative strategies of hormonal therapy, which are used far less frequently in the United States, include ovarian ablation through surgery, radiation therapy to the ovaries, or chemical suppression of ovarian function. Ovarian ablation appears to produce a similar benefit to some chemotherapy regimens. To date, adding ovarian ablation to chemotherapy has not been shown to provide an additional advantage. The value of combining hormonal therapies has not yet been explored adequately.

Hormonal adjuvant therapy should not be recommended to women whose breast cancers do not express hormone receptor protein. Randomized clinical trials have not yet shown that such treatment substantially reduces the likelihood of recurrence or, in the case of tamoxifen, diminishes the likelihood of contralateral breast cancer.

3) FOR WHICH PATIENTS SHOULD ADJUVANT CHEMOTHERAPY BE RECOMMENDED? WHICH AGENTS SHOULD BE USED AND AT WHAT DOSE OR SCHEDULE?

During the past decade, data have emerged that more clearly define the subpopulations of women with localized breast cancer for whom adjuvant chemotherapy is indicated as a standard component of treatment. Chemotherapy has been shown to improve substantially the long-term, relapse-free, and overall survival in both premenopausal and postmenopausal women up to age 70 years with lymph node-positive and lymph node-negative disease.

Randomized clinical trials have attempted to define optimal chemotherapy regimens, doses, and schedules in the adjuvant treatment of breast cancer. These studies, along with the results of overview analyses, permit a number of conclusions to be drawn.

The administration of polychemotherapy (two or more agents) is superior to the administration of single agents. Four to six courses of treatment (3–6 months) appear to provide optimal benefit, with the administration of additional courses adding to toxicity without substantially improving overall outcome. However, definitive data on the benefits of more prolonged treatment are lacking, and future research is needed to address directly this clinically relevant issue.

Anthracyclines, such as doxorubicin and epirubicin, have been used as components of adjuvant polychemotherapy for breast cancer. Available data indicate that adjuvant chemotherapy regimens that include an anthracycline result in a small but statistically significant improvement in survival compared with non-anthracycline-containing regimens. There is no evidence for excessive cardiac toxicity in women without significant pre-existing heart disease treated with anthracyclines at the cumulative doses utilized in standard adjuvant programs. In clinical practice, the decision to use an anthracycline in an individual patient should take into consideration the potential survival benefits versus the specific concern about additional toxicity.

Randomized trials have demonstrated threshold dose effects for two of the most active chemotherapeutic agents, doxorubicin (A) and cyclophosphamide (C). These two drugs are frequently administered together (AC) and appear to result in a comparable survival outcome, whether given preoperatively or postoperatively. However, AC has not been compared with cyclophosphamide/doxorubicin/5-fluorouracil (CAF) or cyclophosphamide/epirubicin/5-fluorouracil (CEF). There is a need for future studies to address the issue of defining the optimal use of anthracycline-based therapy.

There is currently no convincing evidence to demonstrate that more dose-intensive treatment regimens (e.g., high-dose chemotherapy with peripheral stem cell support) result in improved outcomes compared with the administration of polychemotherapy programs at standard-dose levels. Such stem cell-support treatment strategies should not be offered outside the setting of a randomized clinical trial.

Taxanes (docetaxel and paclitaxel) have recently been demonstrated to be among the most active agents in the treatment of metastatic breast cancer. As a result, several studies have explored the clinical utility of adding these drugs to standard AC treatment programs in the adjuvant treatment of lymph node-positive, localized breast cancer. Although a number of such trials have completed accrual and others remain in progress, currently available data are inconclusive and do not permit definitive recommendations regarding the impact of taxanes on either relapse-free or overall survival. There is no evidence to support the use of taxanes in lymph node-negative breast cancer outside the setting of a clinical trial.

Available data demonstrate that chemotherapy and tamoxifen are additive in their impact on survival when employed as adjuvant treatment of breast cancer. Therefore, most patients with hormone receptor-positive tumors who are receiving chemotherapy should receive tamoxifen.

At the present time, there are no convincing data to support

the use of any known biologic factor in selecting a specific adjuvant chemotherapy regimen in breast cancer. Future prospective studies are needed to determine if such factors in an individual patient (e.g., HER-2/neu overexpression) should influence the choice of adjuvant cytotoxic therapy.

Despite the favorable impact of adjuvant chemotherapy on long-term survival in patients with breast cancer, it is important to determine whether there are specific patient populations for whom it is reasonable to avoid the administration of cytotoxic chemotherapy. Unfortunately, very limited information is available to answer this important question. On the basis of available data, it is accepted practice to offer cytotoxic chemotherapy to most women with lymph node metastases or with primary breast cancers larger than 1 cm in diameter (both lymph node-negative and lymph node-positive). For women with lymph node-negative cancers smaller than 1 cm in diameter, the decision to consider chemotherapy should be individualized.

Similarly, in patients with small lymph node-negative breast cancers with favorable histologic subtypes, such as tubular and mucinous cancers, retrospective data support long-term survival following primary therapy without the need for adjuvant chemotherapy.

There are limited data to define the optimal use of adjuvant chemotherapy for women more than 70 years of age. It is likely that there is a survival benefit associated with the administration of chemotherapy in this population of patients. There is legitimate concern, however, regarding the toxicity associated with cytotoxic regimens in this population. In addition, existing comorbid medical conditions and mortality from noncancer causes will influence the overall benefits in this group of women. The decision to treat women over the age of 70 years with adjuvant chemotherapy will need to consider these factors. Increased participation of women over the age of 70 years in randomized clinical trials and studies specifically addressing the value and tolerance of adjuvant chemotherapy in these women are urgently needed.

4) FOR WHICH PATIENTS SHOULD POSTMASTECTOMY RADIOTHERAPY BE RECOMMENDED?

The standard of care for breast conservation includes surgery followed by breast radiotherapy. Before the advent of effective adjuvant chemotherapy, postmastectomy radiotherapy was commonly employed. Interest in this approach was revived after several studies identified patient subgroups with 20%–40% rates of locoregional recurrence after mastectomy and chemotherapy. These subgroups, which included women with four or more positive lymph nodes or an advanced primary tumor (a tumor of ≥ 5 cm or a tumor invading the skin or adjacent musculature), were thought most likely to benefit from a course of postmastectomy radiotherapy.

Recent randomized controlled trials have demonstrated superior tumor control and overall survival rates with the addition of postmastectomy radiotherapy. A recent meta-analysis of more than 22 000 women in which adjuvant radiotherapy was compared with no radiotherapy reported an improvement in locoregional tumor control rates from 70% to 90%. This increase resulted in a statistically significant improvement in the overall survival rate and in the disease-specific survival rate in the study population after a follow-up time of 20 years. These findings

lend support to the concept that improving locoregional tumor control rates in breast cancer can lead to an improvement in survival rates.

The potential benefits of postmastectomy radiotherapy must be weighed against both the acute and the long-term side effects of this therapy. The same meta-analysis documented an excess of non-breast cancer deaths, the majority of which were vascular in nature. These deaths were probably related to the high radiotherapy doses received by the heart and great vessels through the use of outdated radiotherapy techniques. Contemporary radiotherapy delivery employing image-based planning has substantially reduced the radiotherapy dose received by these structures. Although the duration of follow-up of women treated with modern radiotherapeutic techniques is more limited, preliminary data show no apparent increase in vascular deaths. Postmastectomy radiotherapy, however, is associated with an increased risk of arm edema.

There is evidence that women with a high risk of locoregional tumor recurrence after mastectomy will benefit from postoperative radiotherapy. This high-risk group includes women with four or more positive lymph nodes or an advanced primary tumor. Postmastectomy radiotherapy must be coordinated with adjuvant multiagent chemotherapy and/or hormonal therapy. Radiotherapy should not be delivered concurrently with anthracycline chemotherapy and should be delivered within the first 6 months after mastectomy. In most circumstances, combined modality adjuvant therapy begins with several courses of chemotherapy. Radiotherapy, as part of such treatment programs, should be delivered with modern techniques designed to reduce the volume of heart and great vessels receiving radiotherapy. At this time, the role of postmastectomy radiotherapy for women with one to three positive lymph nodes remains uncertain and is being examined in a randomized clinical trial.

5) HOW DO SIDE EFFECTS AND QUALITY-OF-LIFE ISSUES FACTOR INTO INDIVIDUAL DECISION-MAKING ABOUT ADJUVANT THERAPY?

Adjuvant therapy decisions are complicated by marginal differences in treatment results and risk–benefit profiles, balancing acute effects with long-term outcomes. Individual patients differ in the value that they place on these issues. Retrospective studies report that women may be willing to undergo treatment for as little as a 1%–2% improvement in the probability of survival. Clear communication of benefits and risks is an essential component in enabling an informed joint treatment decision as possible. Absolute and relative benefits and risks of therapy must be discussed openly.

Acute, Long-Term, and Late Medical Effects of Adjuvant Therapy

Adjuvant chemotherapy. Studies to date have documented a range of acute and late side effects of adjuvant chemotherapy that have the potential to substantially affect patients' quality of life. Most acute side effects (e.g., nausea and vomiting, mucositis, hair loss, and neutropenia) occur in varying degrees with the different chemotherapy regimens and resolve after treatment completion. The level of psychologic distress associated with chemotherapy also seems to vary between patients. Several randomized studies have found that the psychologic distress that

patients experience is greater during more toxic adjuvant chemotherapy treatment, resolving soon after treatment completion. Similarly, 1–3 years after completing treatment, cancer survivors who had undergone any of the different adjuvant chemohormonal therapies have distress levels equal to those of cancer survivors who had received no further adjuvant therapy.

The simultaneous combination of chemotherapy plus tamoxifen is associated with an increased risk of thromboembolism when compared with tamoxifen alone. Premature menopause, weight gain, and fatigue are the most frequent long- and short-term problems that have been documented. Several small studies have documented mild cognitive problems, such as those in memory, with precise levels of prevalence and severity yet to be determined. There is also a very small increase in the risk of treatment-related second malignancies and cardiac disease.

Adjuvant hormone therapy: tamoxifen and ovarian ablation. Hot flashes and vaginal discharge have been the most common side effects attributed to tamoxifen. Tamoxifen is associated with a small increased risk of endometrial cancer, pulmonary emboli, and deep vein thrombosis, particularly for women 50 years of age or older. The benefits of tamoxifen, however, far outweigh the risks. Tamoxifen has not been associated with an increase in depression, weight gain, nausea and vomiting, diarrhea, or problems in sexual functioning.

As with adjuvant chemotherapy, ovarian ablation is associated with the development of premature menopause and its associated symptoms including osteoporosis.

Decision-making in Adjuvant Therapy for Breast Cancer

Communication between patients and their physicians is the primary vehicle through which complex treatment decisions are made. This communication will likely be facilitated through the use of decision aids and well-designed patient information materials about the medical condition or procedure, treatment side effects, probabilities associated with health outcomes, and impact on quality of life. Findings from current research suggest that decision aids improve patients' knowledge about treatment options, reduce their anxiety about treatment decisions, enhance their comfort with treatment choices, and stimulate the patients to play a more active role in joint decision-making with their physicians.

6) WHAT ARE PROMISING NEW RESEARCH DIRECTIONS FOR ADJUVANT THERAPY?

During the past decade, major advances in adjuvant treatment of breast cancer have resulted from analyses of large prospective randomized trials. In the United States, however, fewer than 3% of cancer patients are entered in clinical trials. To achieve continued improvements in adjuvant treatment, efforts should be made to improve patient and physician participation in these studies. A number of important questions remain to be answered.

Randomized clinical trials should be conducted to better define the risks and benefits of continuing tamoxifen therapy beyond 5 years. Studies are also needed to expand experience with ovarian ablation, to explore the value of combined hormonal therapy, and to determine whether optimal hormonal therapy is equivalent, superior, or additive to chemotherapy in premenopausal women whose tumors express hormone receptor protein. The risks and benefits of new, selective estrogen receptor modu-

lators and aromatase inhibitors should also be examined in the adjuvant setting.

Randomized clinical trials evaluating the roles of high-dose chemotherapy and taxanes need to be completed to determine whether these treatments have a role in the standard management of breast cancer. Additional studies are also needed to determine the importance of variations in the doses and schedules of the drugs used in chemotherapy regimens that are currently being accepted as standard. A particular emphasis should be placed on carefully designed studies to elucidate the clinical and biologic characteristics that may more accurately predict the effectiveness of specific adjuvant treatments in individual patients. As yet unproven treatments that must be critically evaluated in prospective trials in the adjuvant setting include trastuzumab, bisphosphonates, and newer chemotherapeutic and biologic agents.

To date, prospective trials of adjuvant therapy have failed to include sufficient numbers of women older than 70 years. Studies need to be designed that will determine the effectiveness of adjuvant therapies in this group of women.

The role of postmastectomy radiotherapy in women with one to three positive lymph nodes needs to be determined. Investigators should continue to explore the importance of risk factors for recurrence after mastectomy to improve the selection of patients who may benefit from adjuvant radiotherapy. To maximize the possible benefit of adjuvant radiotherapy, new radiation techniques should be developed that further reduce the radiation dose to normal tissues, such as the heart and lungs.

Although adjuvant therapy has been found to produce statistically significant improvements in survival, the ability to predict the value of these treatments in individual patients is limited. The development of accurate predictors of treatment efficacy would permit better targeting of treatments, improving efficacy and reducing the morbidity and cost of treatment. It is essential that the value of predictive and prognostic factors be evaluated with the use of standardized protocols in well-designed clinical studies with sufficient statistical power to detect clinically important differences. Successful integration of new technologies, such as tissue and expression microarrays and proteomics, will depend on careful design and analysis of clinical investigations. The value of sentinel lymph node biopsy and of sensitive assays for micrometastatic disease in lymph nodes and bone marrow should also be important priorities for clinical research.

Quality-of-life and late-effect evaluations should be integrated judiciously into selected clinical trials to better discern the acute and long-term influence of treatment on patients and their families. Interventions should be sought that will reduce side effects and improve quality of life. Decision aids and other techniques should be developed and evaluated for their ability to improve patients' involvement and understanding of treatment decisions.

CONCLUSIONS

During the past 10 years, substantial progress has been made in the treatment of breast cancer. For the first time, mortality rates for breast cancer are decreasing in the United States. Refinements of adjuvant treatment have contributed to this advance.

Generally accepted prognostic and predictive factors include age, tumor size, lymph node status, histologic tumor type, grade,

mitotic rate, and hormone receptor status. Novel technologies, such as tissue and expression microarrays and proteomics, hold exciting potential. Progress, however, will depend on proper design and analysis of clinical and pathologic investigations.

Decisions regarding adjuvant hormonal therapy should be based on the presence of hormone receptor protein in tumor tissues. Adjuvant hormonal therapy should be offered to women whose tumors express hormone receptor protein. At present, 5 years of tamoxifen is standard adjuvant hormone therapy; ovarian ablation represents an alternative to adjuvant hormonal therapy for selected premenopausal women. Adjuvant hormonal therapy should not be recommended to women whose tumors do not express hormone receptor protein.

Because adjuvant polychemotherapy improves survival, it should be recommended to the majority of women with localized breast cancer regardless of lymph node, menopausal, or hormone receptor status. The inclusion of anthracyclines in adjuvant chemotherapy regimens produces a small but statistically significant improvement in survival over non-anthracycline-containing regimens.

Available data are currently inconclusive regarding the use of taxanes in adjuvant treatment of lymph node-positive breast cancer. The use of adjuvant dose-intensive chemotherapy regimens

in high-risk breast cancer and of taxanes in lymph node-negative breast cancer should be restricted to randomized trials. Ongoing studies evaluating these treatment strategies should be supported to determine if such strategies have a role in adjuvant treatment. Studies to date have included few patients older than 70 years. There is a critical need for trials to evaluate the role of adjuvant chemotherapy in these women.

There is evidence that women with a high risk of locoregional tumor recurrence after mastectomy benefit from postoperative radiotherapy. This high-risk group includes women with four or more positive lymph nodes or an advanced primary cancer. Currently, the role of postmastectomy radiotherapy for patients with one to three positive lymph nodes remains uncertain and should be tested in a randomized controlled trial.

Individual patients differ in the importance that they place on the risks and benefits of adjuvant treatments. Quality of life needs to be evaluated in selected randomized clinical trials to examine the impact of the major acute and long-term side effects of adjuvant treatments, particularly premature menopause, weight gain, mild memory loss, and fatigue. Methods to support shared decision-making between patients and their physicians have been successful in trials; they need to be tailored for diverse populations and should be tested for broader dissemination.

CONSENSUS DEVELOPMENT PANEL

Patricia Eifel, M.D.
Panel and Conference Chairperson
Professor of Radiation Oncology
The University of Texas M. D. Anderson Cancer
Center
Houston, TX

John A. Axelson, M.D., FACP
Hematology and Oncology Associates
Jackson, MI

Jose Costa, M.D.
Professor of Pathology and Biology
Director of Anatomic Pathology
Deputy Director, Yale Cancer Center
Vice Chairman, Department of Pathology
Yale University School of Medicine
New Haven, CT

John Crowley, Ph.D.
Biostatistician
Fred Hutchinson Cancer Research Center
Seattle, WA

Walter J. Curran, Jr., M.D.
Professor and Chairman
Department of Radiation Oncology
Thomas Jefferson University Hospital
Philadelphia, PA

Ann Deshler, R.N.
Administrative Director
Metro Minnesota Community Clinical Oncology
Program
Institute for Research and Education of Health
System Minnesota
St. Louis Park, MN

Shirley Fulton, J.D., M.B.A.
Superior Court Judge
Superior Court Judge Office
Charlotte, NC

Carolyn B. Hendricks, M.D.
Medical Oncologist
Suburban Specialty Care Physicians, P.C.
Bethesda, MD

Margaret Kemeny, M.D.
Surgeon
Chief of the Division of Surgical Oncology
University Hospital and Medical Center
State University of New York at Stony Brook
Stony Brook, NY

Alice B. Kornblith, Ph.D.
Director of Outcomes Studies
Department of Pain Medicine and Palliative Care
and Cancer Center
Beth Israel Medical Center
New York, NY

Thomas A. Louis, Ph.D.
Senior Statistical Scientist
The RAND Corporation
Arlington, VA

Maurie Markman, M.D.
Director, The Cleveland Clinic
Taussig Cancer Center
Chairman, Department of Hematology and
Medical Oncology
The Lee and Jerome Burkons Research Chair in
Oncology
The Cleveland Clinic Foundation
Cleveland, OH

Robert Mayer, M.D.
Professor of Medicine
Harvard Medical School
Vice Chair for Academic Affairs
Department of Adult Oncology
Dana-Farber Cancer Institute
Boston, MA

Debra Roter, Dr.P.H.
Professor, Health Policy and Management

School of Hygiene and Public Health
The Johns Hopkins University
Baltimore, MD

SPEAKERS

Karen H. Antman, M.D.
Professor of Medicine
College of Physicians and Surgeons of Columbia
University
Chief, Division of Medical Oncology
Director, Herbert Irving Comprehensive Cancer
Center
New York, NY

Jonas C. Bergh, M.D., Ph.D.
Professor of Clinical and Molecular Oncology
Karolinska Institute and Hospital
Stockholm, Sweden

John L. Bryant, Ph.D.
Associate Professor of Biostatistics
University of Pittsburgh
Director, Biostatistical Center
National Surgical Adjuvant Breast and Bowel
Project
Pittsburgh, PA

Gary M. Clark, Ph.D.
Professor of Medicine
Baylor Breast Center
Baylor College of Medicine
Houston, TX

Alan Coates, M.D., FRACP
International Breast Cancer Study Group
Chief Executive Officer
Australian Cancer Society
Sydney, New South Wales, Australia

Jack Cuzick, Ph.D.
Professor of Epidemiology
Head, Department of Mathematics, Statistics, and
Epidemiology

Imperial Cancer Research Fund
London, U.K.

Maria Grazia Daidone, Ph.D.
Unit 10 Determinants of Prognosis and Treatment
Response
Department of Experimental Oncology
Istituto Nazionale Tumori
Milan, Italy

Nancy E. Davidson, M.D.
Professor
The Johns Hopkins Oncology Center
The Johns Hopkins University School of Medicine
Baltimore, MD

Christina Davies, MBChB, M.Sc.
ATLAS Coordinator
Clinical Trial Service Unit
Radcliffe Infirmary
University of Oxford
Oxford, U.K.

James J. Dignam, Ph.D.
Statistician
National Surgical Adjuvant Breast and Bowel
Project
Chicago, IL

Bernard Fisher, M.D.
Scientific Director
National Surgical Adjuvant Breast and Bowel
Project
Distinguished Service Professor
University of Pittsburgh
Pittsburgh, PA

Patricia A. Ganz, M.D.
Professor, University of California, Los Angeles,
Schools of Medicine and Public Health
Director, Division of Cancer Prevention and
Control Research
Jonsson Comprehensive Cancer Center
Los Angeles, CA

Aron Goldhirsch, M.D.
Chairman, Scientific Committee, International
Breast Cancer Study Group
Professor of Medical Oncology
Director, Division of Medical Oncology
European Institute of Oncology
Milan, Italy

Richard Gray, M.A., M.Sc.
Director
Clinical Trials Unit
University of Birmingham Medical School
Birmingham, U.K.

I. Craig Henderson, M.D.
Adjunct Professor of Medicine
University of California, San Francisco
San Francisco, CA

Gabriel N. Hortobagyi, M.D., FACP
Professor and Chairman
Department of Breast Medical Oncology
The University of Texas M. D. Anderson Cancer
Center
Houston, TX

Amy S. Langer, M.B.A.
Executive Director
National Alliance of Breast Cancer Organizations
New York, NY

Mark Norman Levine, M.D.
Professor of Medicine
McMaster University
Hamilton, ON, Canada

Eleftherios P. Mamounas, M.D.
Medical Director
Cancer Center
Aultman Hospital
Canton, OH

Monica Morrow, M.D.
Professor of Surgery, Northwestern Memorial
Hospital
Northwestern University Medical School
Director, Lynn Sage Comprehensive
Breast Program
Director of Cancer Department
American College of Surgeons
Chicago, IL

Hyman B. Muss, M.D.
Associate Director, Vermont Cancer Center
Professor of Medicine, University of Vermont
College of Medicine
Director of Hematology/Oncology
Fletcher Allen Health Care
University of Vermont
Burlington, VT

Larry Norton, M.D.
Head, Division of Solid Tumor Oncology
Norna S. Sarofim Chair in Clinical Oncology
Memorial Sloan-Kettering Cancer Center
New York, NY

C. Kent Osborne, M.D.
Professor
Baylor Breast Center
Baylor College of Medicine
Houston, TX

William P. Peters, M.D., Ph.D.
Director and Chief Executive Officer
Barbara Ann Karmanos Cancer Institute
Detroit, MI

Sir Richard Peto, F.R.S., M.Sc.
Early Breast Cancer Trialists'
Collaborative Group Secretariat
Professor of Medical Statistics and Epidemiology
Co-Director
Imperial Cancer Research Fund/Medical Research
Council Clinical Trial Service Unit and
Epidemiological Studies Unit
Radcliffe Infirmary, University of Oxford
Oxford, U.K.

Martine J. Piccart, M.D., Ph.D.
Chairman, Breast International Group
Head, Chemotherapy Department
Jules Bordet Institute
Brussels, Belgium

Lori Pierce, M.D.
Associate Professor
Department of Radiation Oncology
University of Michigan Medical Center
Ann Arbor, MI

Peter Ravdin, M.D., Ph.D.
Associate Professor
Department of Medicine
Division of Medical Oncology

University of Texas Health Science Center at San
Antonio
San Antonio, TX

Stuart J. Schnitt, M.D.
Associate Professor of Pathology
Harvard Medical School
Director of Surgical Pathology
Beth Israel Deaconess Medical Center
Boston, MA

George W. Sledge, Jr., M.D.
Ballvé-Lantero Professor of Oncology
Department of Medicine
Indiana University School of Medicine
Indianapolis, IN

Eric P. Winer, M.D.
Associate Professor of Medicine
Department of Adult Oncology
Dana-Farber Cancer Institute
Boston, MA

Norman Wolmark, M.D.
Chairman, National Surgical Adjuvant Breast and
Bowel Project
Chairman and Professor
Department of Human Oncology
Allegheny General Hospital
Pittsburgh, PA

William C. Wood, M.D., FACS
Joseph Brown Whitehead Professor and Chairman
Department of Surgery
Emory University School of Medicine
Atlanta, GA

PLANNING COMMITTEE

Jeffrey Abrams, M.D.
Planning Committee Chairperson
Senior Investigator
Clinical Investigation Branch
Cancer Therapy Evaluation Program
Division of Cancer Treatment and Diagnosis
National Cancer Institute
National Institutes of Health
Bethesda, MD

Marietta Anthony, Ph.D.
Director, Women's Health Research
Department of Pharmacology
Georgetown University Medical Center
Washington, DC

Karen H. Antman, M.D.
Professor of Medicine
College of Physicians and Surgeons of Columbia
University
Chief, Division of Medical Oncology
Director, Herbert Irving Comprehensive Cancer
Center
New York, NY

Christine D. Berg, M.D.
Director, Suburban Hospital Cancer Center
Affiliated with The Johns Hopkins Oncology
Center
Bethesda, MD

John A. Bowersox
Communications Specialist
Office of Medical Applications of Research
Office of the Director

National Institutes of Health
Bethesda, MD

John L. Bryant, Ph.D.
Associate Professor of Biostatistics
University of Pittsburgh
Director, Biostatistical Center
National Surgical Adjuvant Breast and Bowel
Project
Pittsburgh, PA

Alan Coates, M.D., FRACP
International Breast Cancer Study Group
Chief Executive Officer
Australian Cancer Society
Sydney, Australia

Nancy E. Davidson, M.D.
Professor
The Johns Hopkins Oncology Center
The Johns Hopkins University School of Medicine
Baltimore, MD

Patricia Eifel, M.D.
Professor of Radiation Oncology
The University of Texas M. D. Anderson Cancer
Center
Houston, TX

Jerry M. Elliott
Program Analysis and Management Officer
Office of Medical Applications of Research
Office of the Director
National Institutes of Health
Bethesda, MD

John H. Ferguson, M.D.
Consultant, Office of Rare Diseases
National Institutes of Health
Bethesda, MD

Patricia A. Ganz, M.D.
Professor, University of California, Los Angeles,
Schools of Medicine and Public Health
Director, Division of Cancer Prevention and
Control Research

Jonsson Comprehensive Cancer Center
Los Angeles, CA

Gabriel N. Hortobagyi, M.D., FACP
Professor and Chairman
Department of Breast Medical Oncology
The University of Texas M. D. Anderson Cancer
Center
Houston, TX

Karen Eubanks Jackson
National President and Founder
Sisters Network, Inc.
Houston, TX

Barnett S. Kramer, M.D., M.P.H.
Director
Office of Medical Applications of Research
Office of the Director
National Institutes of Health
Bethesda, MD

Amy S. Langer, M.B.A.
Executive Director
National Alliance of Breast Cancer Organizations
New York, NY

Daniel J. O'Neal III, R.N., M.A.
Chief
Office of Science Policy and Public Liaison
National Institute of Nursing Research
National Institutes of Health
Bethesda, MD

Lori Pierce, M.D.
Associate Professor
Department of Radiation Oncology
University of Michigan Medical Center
Ann Arbor, MI

Charles R. Sherman, Ph.D.
Deputy Director
Office of Medical Applications of Research
Office of the Director
National Institutes of Health
Bethesda, MD

Sheila E. Taube, Ph.D.
Associate Director of Cancer Diagnosis Program
Division of Cancer Treatment and Diagnosis
National Cancer Institute
National Institutes of Health
Bethesda, MD

Ann Thor, M.D.
Professor
Departments of Pathology and Surgery
Northwestern University Medical School
Evanston Northwestern Healthcare
Evanston, IL

William C. Wood, M.D., FACS
Joseph Brown Whitehead Professor and Chairman
Department of Surgery
Emory University School of Medicine
Atlanta, GA

JoAnne Zujewski, M.D.
Senior Medical Oncologist
Division of Clinical Sciences
National Cancer Institute
National Institutes of Health
Bethesda, MD

CONFERENCE SPONSORS

National Cancer Institute
Richard D. Klausner, M.D.
Director

Office of Medical Applications of Research
Barnett S. Kramer, M.D., M.P.H.
Director

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Director

Office of Research on Women's Health
Vivian W. Pinn, M.D.
Director

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Adjuvant Therapy for Breast Cancer: Current Controversies and Future Prospects

William C. Wood

Carcinoma of the breast remains the most common cancer in women in the United States. It is exceeded only by lung cancer as a cause of cancer death in women. In 1980, the National Institutes of Health (Bethesda, MD) held a consensus development conference that addressed the efficacy of adjuvant chemotherapy in breast cancer (1). In 1985, another conference was held that addressed the survival benefit associated both with adjuvant chemotherapy and with adjuvant hormonal therapy for women with primary breast cancer (2). In 1990, at the time of the last consensus development conference (3), breast cancer appeared to be increasing modestly nationally, partly because of increased detection of early breast cancer by the growing use of screening mammography. The mortality rate in 1990 appeared to be absolutely flat. The consensus conference held 10 years ago concluded that breast conservation therapy was appropriate for the management of primary breast cancer. It further concluded that adjuvant systemic therapy provided benefit to women regardless of lymph node status and that it should be recommended based on risk and benefit as judged by a complex of prognostic factors, including size of the primary tumor and lymph node status. These conclusions were based on large, randomized clinical trials. Every major advance in the management of breast cancer has emerged from clinical trials. Without continued basic research in breast cancer, there will be no future for clinical trials.

At the present time, breast cancer in the United States appears to be at a plateau of frequency when it is age adjusted. The encouraging news is that mortality from breast cancer in this country and in many European countries has been falling for the last 8 years, which probably reflects the benefit of both adjuvant tamoxifen and screening mammography. The last decade has brought major clinical trials addressing the significance of duration in the administration of adjuvant tamoxifen, the role of anthracyclines in adjuvant chemotherapy, the role of taxanes, the significance of chemotherapy dose and intensity, the significance of chemotherapy duration, and predictive factors for response to adjuvant therapy. These clinical trials provide the datasets that enable this conference to address these current controversies.

The questions that have been chosen for the panel to address meet two criteria: 1) They are of crucial interest to breast cancer patients and their healthcare team, and 2) a body of scientific evidence exists to allow an informed consensus.

Several questions were debated and felt to be important future prospects presently not ready for consensus evaluation. One was the mapping and evaluation of sentinel lymph nodes to tailor lymph node staging. Numerous questions are under investigation regarding the type of sentinel lymph node mapping best used, the false-negative rate to be expected, and its relationship to experience. Clinical trials have already demonstrated the role of

surgical experience, both as the percentage of sentinel lymph nodes identified and the false-negative rate. Large randomized clinical trials are under way to address these questions, as well as the optimal site of tracer injection within the breast and the degree to which sentinel lymph nodes are specific to the area of the breast in which the primary tumor arises. Another question involves the role of immunohistochemical evaluation of bone marrow biopsies. Which antibody combination to use and what patients will have an advantage by such information and whether such information will be predictive of therapeutic response or only prognostic are still under evaluation. Either of these assays can be overinterpreted by detecting molecular evidence of tumor cells that are not micrometastases but simply circulating in the process of apoptosis or immune destruction. These are important issues being addressed with current clinical trials.

Herceptin is a potent new agent in the treatment of breast cancer. Its role as a component of adjuvant systemic therapy is being evaluated in large randomized clinical trials by the National Institutes of Health Breast Intergroup, the National Surgical Adjuvant Breast and Bowel Project, the Breast Cancer Treatment Research Group, and the Breast International Group. The significance of bisphosphonates as a component of adjuvant therapy is being studied. The delayed use of adjuvant hormonal therapy or chemotherapy and the use of techniques of maintenance therapy as a component of adjuvant therapy are all undergoing clinical trial at this time. Tailored therapy for locally advanced breast cancer is being addressed in numerous clinics and cooperative groups. The treatment of *in situ* carcinoma and the prophylaxis of persons at high risk are continuing to evolve and may deserve consideration by consensus panels in the future. Other future prospects include the significance of antiangiogenesis therapies as a component of adjuvant therapy, the use of vaccines, tailored small molecules, antisense constructs, and other therapies arising from the rapid advances of tumor biology and molecular genetics. None of these was judged by the planning committee to have sufficient evidence from mature trials for consensus development. Each is worthy of ongoing research.

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Correspondence to: William C. Wood, M.D., Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322

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Interpreting and Integrating Risk Factors for Patients With Primary Breast Cancer

Gary M. Clark

The term risk factor has different meanings in different contexts. Some factors may be patient specific (e.g., race, age, socioeconomic status, and environment), while others may be disease specific (e.g., biomarkers measured on tumor specimens, serum, and bone marrow). These factors have several potential clinical uses, including diagnosing a disease or assessing the risk of developing a disease, estimating prognosis for patients diagnosed with a specific disease who receive no therapy, predicting response to a particular therapy, monitoring response to therapy during a treatment course, and identifying targets of opportunity for new therapies. This article focuses on prognostic and predictive biomarkers and provides guidelines for interpreting published reports about these biomarkers. Application of these guidelines to the assessment of micrometastases in bone marrow of patients with breast cancer yields the conclusion that standardized techniques that are sensitive and reproducible for detecting micrometastases are needed before we can evaluate their prognostic significance. [J Natl Cancer Inst Monogr 2001;30:17-21]

The primary objective of this presentation is to give members of the Consensus Development Panel and readers of the scientific literature some guidelines for interpreting published reports about biomarkers. These guidelines are then applied to the assessment of micrometastases in bone marrow of patients with breast cancer.

CLINICAL USES FOR BIOMARKERS

There are several potential clinical uses for biomarkers (Table 1). Biomarkers may aid in diagnosing a disease or in assessing the risk of developing a disease; good examples of this are PSA for prostate cancer and BRCA1 or BRCA2 for breast cancer. Validation of these types of biomarkers requires assessment of patients with and without the disease. Biomarkers may also be useful for estimating the prognosis or natural history of a disease. The commonly used staging systems for various types of cancer are based on these types of factors. For breast cancer, these include axillary lymph node status, tumor size, and tumor grade. Validation of these biomarkers requires assessment of patients with a specific cancer who received no therapy or, possibly, a standard therapy, and correlation between marker status and clinical outcome. Biomarkers may also be used to predict response to particular therapies. Estrogen receptor (ER) and progesterone receptor (PgR) are biomarkers that predict response to hormonal therapy in both the adjuvant setting and in metastatic disease. Validation of these types of biomarkers generally requires patients who were randomly assigned to receive the particular therapy or no therapy to minimize patient selection biases that may be associated with marker status. Biomarkers may also be used to monitor response to therapy. The surface markers CA 125, CA 15.3, and carcinoembryonic antigens (CEAs) are good

examples of these types of factors. Regardless of other clinical uses, biomarkers might be targets for new therapies. The HER-2/neu oncogene led to the development and approval of trastuzumab (Herceptin) for patients with breast cancer whose tumors overexpress this biomarker.

Although the clinical uses for biomarkers are quite varied, there are many common features that should be considered when evaluating biomarker studies. First, there should be a biologic hypothesis that underlies the study. What is the evidence that this biomarker might be a diagnostic, prognostic, or predictive factor? The authors should state whether their study is a first-generation pilot study or a definitive study designed to confirm previous findings. All too often a study that was designed as a pilot or feasibility study becomes a definitive study when the first paper is published. If this is a definitive study, then there should be sufficient sample size to address the question and the biomarker should be analyzed by using both univariate and multivariate techniques that include other established factors. The assay used to assess biomarker status should be validated before conducting the study, and the definition of a positive assay or an abnormal result should be stated clearly. Before new biomarkers are recommended for routine clinical practice, the assay must be shown to be reproducible, and the results must be shown to be able to be generalized to other sets of patients.

COMMON CLINICAL ENDPOINTS FOR BIOMARKER STUDIES

Interpretation of biomarker studies requires a clear understanding of the clinical endpoints that were used in the studies. Some commonly used endpoints include overall survival, breast cancer-specific survival, relative survival, disease-free survival, progression-free survival, event-free survival, tumor response, and modulation of a biomarker. Some of these endpoints are clear and unambiguous—for example, overall survival. Other endpoints require explicit definition within the publication. For example, what types of events are included in event-free survival? Does disease-free survival include both local and distant recurrences? Does it include contralateral breast cancers and death caused by breast cancer or other causes? Breast cancer-specific survival requires ascertainment of cause of death. Given the notorious unreliability of cause of death given on death certificates (1,2), authors should state how causes of death were determined in their studies. Tumor response has been standardized, and most authors use a common definition, but some publications include stable disease as a response and others do not. The Southwest Oncology Group has published their criteria for the clinical endpoints that are used in their therapeutic studies

Correspondence to: Gary M. Clark, Ph.D., Breast Center at Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 (e-mail: gmclark@breastcenter.tmc.edu).

See "Note" following "References."

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Table 1. Clinical uses for biomarkers*

Type	Examples
Diagnosis/risk assessment	PSA, BRCA1, BRCA2
Prognosis/natural history/staging	Lymph node status, tumor size
Predicting response to therapy	ER, PgR
Monitoring response to therapy	CA 125, CA 15.3, CEA
Targets for therapy	HER-2 neu

*PSA = prostate-specific antigen; ER = estrogen receptor, PgR = progesterone receptor.

(3). These criteria might form the basis for more standardized reporting of clinical outcomes in the future.

There are several different ways to summarize and report clinical outcomes. For example, comparisons of mortality between groups of patients can be presented as the absolute risk difference, the relative risk, or an odds ratio. Each is a legitimate summary statistic, but the same statistic must be used to compare different studies. The absolute difference is easy to interpret, but its impact depends on the individual mortality rates in each group. The relative risk is defined as the risk of dying in the treated group divided by the risk of dying in the control group. It is often estimated by the hazard ratio from a statistical model such as the Cox proportional hazards model (4). The odds ratio is the odds of dying versus the odds of surviving in the treated group divided by the odds of dying versus those of surviving in the control group. Suppose that a control group and a treated group each contains 100 patients. If 50 patients in the control group died but only 10 patients in the treated group died, the absolute risk difference would be 40%. The relative risk of dying is (10 of 100)/(50 of 100), or 0.20. The odds ratio is (10 of 90)/(50 of 50), or 0.11. Table 2 gives examples of these statistics under three different scenarios.

PROGNOSTIC VERSUS PREDICTIVE BIOMARKERS

Discussion of risk factors for making treatment decisions about adjuvant therapy usually focuses on prognostic biomarkers and predictive biomarkers. It is important to distinguish between these types of factors. I have previously defined a prognostic factor for primary breast cancer as any measurement available at the time of diagnosis or surgery that is associated with disease-free or overall survival in the absence of systemic adjuvant therapy (5,6). Note that this definition permits application of a standard therapy (e.g., surgery) that all patients are likely to receive. A predictive factor is any measurement associated with response or lack of response to a particular therapy. Response can be defined by using any of the clinical endpoints described above.

The graphic examples in Fig. 1 help to illustrate the differences between prognostic factors and predictive factors. The lower line in Fig. 1, A, demonstrates that axillary lymph node status is a prognostic factor. Untreated patients with negative lymph nodes who receive no adjuvant therapy have a better

clinical outcome than untreated patients with positive lymph nodes. If patients are treated and the improvement in survival is the same for lymph node-negative and lymph node-positive patients, as shown in Fig. 1, A, then lymph node status does not differentially predict which patients will benefit from therapy. Thus, the lines are parallel and the biomarker is not a predictive factor. This is consistent with results from the overview analyses that have shown that the reduction in odds of death for either chemotherapy (7) or hormonal therapy (8) is the same for lymph node-negative and lymph node-positive patients.

If untreated patients with ER-positive (ER⁺) tumors have the same clinical outcome as untreated patients with ER-negative (ER⁻) tumors, as shown in Fig. 1, B, then ER status would not be a prognostic factor. But it is well known that ER⁺ tumors respond much better to hormonal therapy than do ER⁻ tumors, so we see a differential response to hormonal therapy. If, however, untreated patients with ER⁺ tumors have a better prognosis than untreated patients with ER⁻ tumors, as shown in Fig. 1, C, then ER status would be both prognostic and predictive. Another biomarker that is probably both prognostic and predictive is HER-2/neu status. Untreated patients with HER-2/neu-negative tumors probably have a slightly better prognosis than untreated patients with HER-2/neu-positive tumors, as shown in Fig. 1, D, especially if they are lymph node-positive. If a treatment such as Herceptin is very effective for HER-2/neu-positive tumors but has little or no effect on HER-2/neu-negative tumors, we might have the scenario shown in Fig. 1, D, where the prognostic and predictive effects move in opposite directions.

The common feature in Fig. 1, B–D, that demonstrates a predictive effect is the differential response to therapy represented by nonparallel lines. In statistical terms, this constitutes an interaction between response to treatment and biomarker status. When an interaction exists and investigators combine treated and untreated patients in analyses of potential prognostic factors, it is difficult, if not impossible, to separate the predictive effects from the prognostic effects. Unless these effects are separated, it is very difficult to make treatment recommendations based on the status of the biomarker. How can we recommend a particular therapy if we do not know if the expected good outcome will be caused by effective therapy or by the presence of a good prognostic factor?

FALSE-NEGATIVE AND FALSE-POSITIVE STUDIES

Evaluation and interpretation of the published literature dealing with biomarkers is fraught with difficulties. Many reported biomarker results are either falsely negative or falsely positive.

Statistical power is the probability of detecting an effect when it really exists. Increasing the sample size increases power. Most studies of potential prognostic or predictive factors are substantially underpowered, so that a negative result may reflect either a small or nonexistent marker effect or a lack of statistical power. Evaluation of a potential predictive biomarker is much more difficult than evaluation of a new therapy. Biomarker status cannot be randomized, and imbalance must be taken into account. A test for an interaction between response to treatment and biomarker status can require up to four times more events than a test for a treatment main effect (9). Biomarker studies are often conducted as ancillary studies to already completed, randomized clinical trials that were designed to compare treatments. The subsets of patients who do or do not have the biomarker are much smaller than the randomized groups who received the

Table 2. Summary statistics for mortality

Mortality	Absolute risk difference, %	Relative risk	Odds ratio
10% vs. 50%	40	0.20	0.11
5% vs. 20%	16	0.20	0.17
2% vs. 10%	8	0.20	0.18

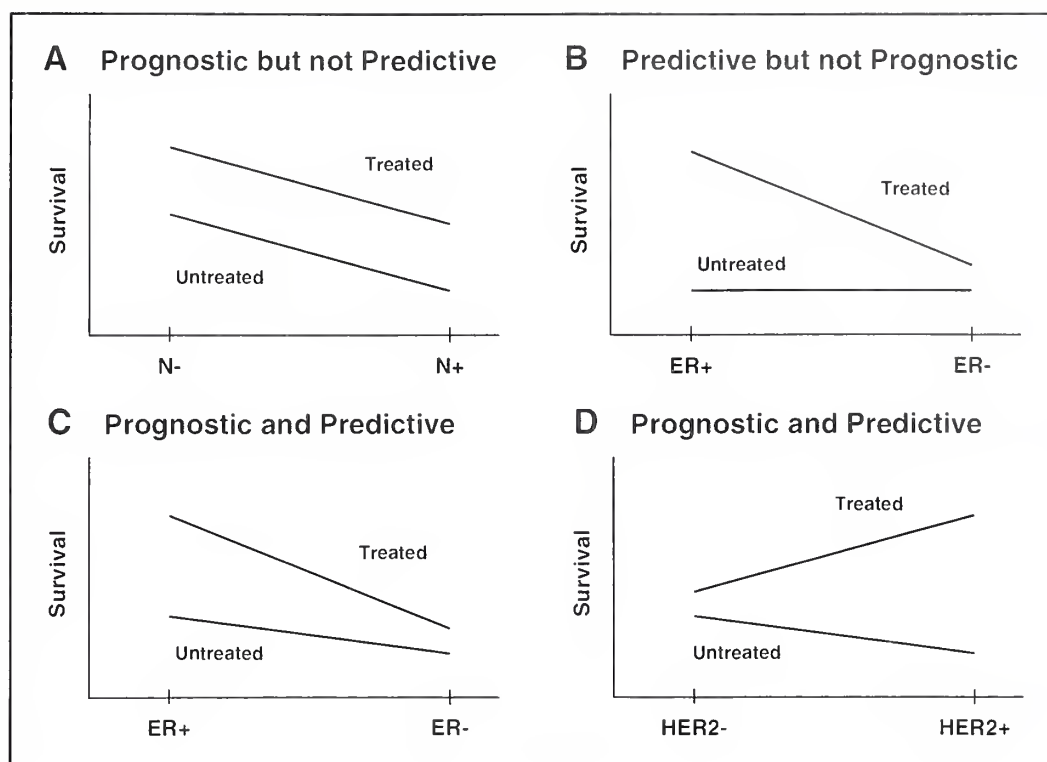


Fig. 1. Examples of prognostic and/or predictive biomarkers.

treatments, so statistical power is greatly reduced. An additional consideration is that many biomarker studies require retrieval of paraffin blocks or other archived tissues for the assessment of biomarker status. The combined rate of specimen retrieval and assay evaluability is often less than 50% of the patients who participated in the clinical trial. Therefore, most studies of potential predictive biomarkers will produce false-negative results.

However, many of the results that appear in the literature about biomarkers are probably false-positive results. Publication bias is a well-known phenomenon. It is much easier to publish an apparent positive result than a negative result. Results may appear to be positive as a consequence of multiple tests of hypotheses. If we perform enough subset analyses, sooner or later one of these analyses will produce statistically significant results by chance alone. In the case of biomarkers, a common problem is the identification of a cut point that separates biomarker results into high- and low-risk categories. Searches for an optimal cut point are simply multiple tests of hypotheses that inevitably lead to significant results, and adjustments must be made before accepting these positive findings (10). Other problems include analysis of many potential biomarkers but publication of only the significant factors and analysis of multiple subsets of patients but conclusions based only on the significant results.

It is necessary to validate the results of apparently positive biomarker studies. This validation should include the use of standardized, reproducible assays with predefined scoring systems and cut points. A common mistake is to assess biomarker status in an additional cohort of patients, combine these patients with the initial cohort, and perform a single analysis. The new cohort must first be analyzed as a stand-alone validation set that is not contaminated with the overly optimistic results obtained in the original cohort after tests of multiple hypotheses. It may or may not be appropriate to combine the cohorts after this initial analysis. The literature is replete with multiple reports from the

same investigators who included subsets of the same patients in analyses that were intended to validate their initial findings.

MICROMETASTASES IN BONE MARROW

The Consensus Development Panel asked for an assessment of the published literature concerning the clinical utility of micrometastases in the bone marrow of patients with breast cancer. Three specific questions are asked: 1) What is the prevalence of micrometastases in primary breast cancer? 2) Is the presence of micrometastases associated with known prognostic factors? 3) Does the presence of micrometastases predict clinical outcome?

Two excellent reviews (11,12) of this topic were published in 1998. Each article reviewed 11 studies, with eight in common. Subsequent to these publications, at least three additional studies (13–15) have been reported. It is immediately clear that there are many methodologic differences among these 17 published studies. Some used smears and others used cytospin preparations, the number of cells evaluated differed considerably and was often not reported; at least 23 different antibodies were used (antiepithelial cell-surface antigens, anti-milk fat globulins, anticytokeratin components, and antipolymorphic epithelial mucins); different staining procedures were used; some used healthy control subjects to define cut points and others used arbitrary definitions for the presence of micrometastases; and demographic and tumor characteristics of the patients differed among studies. If we ignore these methodologic differences, the weighted average of the prevalence of micrometastases in these 17 studies was 32% (range, 1%–49%).

An obvious question when interpreting this result is, What is the impact of using different antibodies to detect micrometastases? Braun and Pantel (12) described differential immunohistochemical staining of bone marrow from noncancer patients using

a panel of antibodies (Table 3). These differences could have a major impact on the reported prevalences in the published studies. The inclusion of patients with different demographic and tumor characteristics could also bias this estimate of prevalence. To partially address this question, each published study was reviewed to determine whether correlations with other factors were examined and, if so, whether significant relationships were found (Table 4). The conclusions based on this review are less than satisfactory. Only a minority of the studies attempted to associate the presence of micrometastases in bone marrow with other prognostic factors. Of those that did, only five of 11 found a relationship with axillary lymph node status by routine hematoxylin-eosin evaluation, and three of nine found a relationship with tumor size. Thus, the answer to the second question about relationships with other prognostic factors is unclear.

It is difficult to determine from the published literature if the presence of micrometastases in bone marrow correlates with clinical outcomes. Most of these studies included univariate analyses with either disease-free or overall survival as the clinical endpoint. Only nine of the 17 studies performed multivariate analyses; three reported a significant association with disease-free survival, and five reported a relationship with overall survival. However, almost all studies included treated and untreated patients. The untreated subsets were generally too small for definitive analyses, and the treated subsets received a variety of heterogeneous therapies. None of the studies performed tests of interactions between response to treatment and the presence of micrometastases. Therefore, it is very difficult to separate the predictive effects from the prognostic effects in any of these studies.

The overall conclusion of this exercise is that meta-analyses of these types of biomarker studies are not appropriate. The differences in detection techniques, sensitivities, and specificities of the assays, patient characteristics, and treatments produce too much heterogeneity among studies to combine their results. To evaluate the prognostic significance of micrometastases, we need well-designed, prospective studies that use sensitive and reproducible standardized techniques for detecting micrometastases. The recent publication by Braun et al. (15) is a step in the right direction; however, we need additional studies to confirm their impressive findings.

SUMMARY

The evaluation of biomarkers as prognostic or predictive factors requires the development of standardized and validated assays. It requires study designs that differentiate between prognostic and predictive effects. These studies should have clear eligibility criteria and sufficient numbers of patients and tissues to answer clinically relevant questions with adequate statistical power. We should require a high level of evidence before in-

Table 3. Immunohistochemical staining of bone marrow from noncancer patients*

Antibody	Antigen	Percentage positive
A45-B/B3	CK	0.9
CK2	CK18	2.8
E29	EMA	26.7
HMF/G1	HMF/G	42.7
2E11	TAG-12	54.3

*Modified from Braun and Pantel (12).

Table 4. Correlations between micrometastases in bone marrow and other prognostic factors (based on 17 published studies)

Factor	No. of positive studies/total No.
Lymph node	5/11
Tumor size	3/9
Estrogen receptor status	1/7
Menopausal status	1/7
Tumor type	3/5
Tumor grade	2/4

corporating new biomarkers into clinical practice (16). Criteria for determining the clinical utility of biomarkers have only recently been proposed. The American Society of Clinical Oncology used very conservative criteria to develop practice guidelines for using biomarkers (17). Partly in response to the lack of consensus about these criteria, the Tumor Marker Utility Grading System was developed to differentiate levels of evidence among published studies (18). The College of American Pathologists used a modification of this system to develop their consensus statements about prognostic factors in breast, colon, and prostate cancers (19).

Study designs to evaluate biomarkers for different clinical uses vary with respect to the types of subjects and/or tissues that need to be studied, the endpoints that need to be measured, and the number of subjects and/or tissues that need to be accrued. However, the basic methodologic principles for good study designs are common to all clinical uses. All study designs should be based on clearly stated hypotheses. Assays should be reproducible and should be performed without knowledge of the clinical data and patient outcome. Results for individual factors should be analyzed by use of multivariate techniques that incorporate standard biomarkers that are already in clinical use. All results should be validated in subsequent studies before they are incorporated into clinical practice.

Very few new prognostic or predictive factors have been validated and endorsed for clinical use during the past several years. Part of the reason is a lack of adherence to proposed guidelines for the design, conduct, analysis, and reporting of results from prognostic factor studies (20). It is time to translate the principles of good study design and analysis that have been developed for clinical trials to the evaluation of new biomarkers.

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NOTE

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There has long been a pressing clinical need to identify prognostic and predictive factors for patients with breast cancer. Although numerous candidate biological and molecular markers have been identified during the last two decades, traditional factors such as lymph node status, tumor size, histologic type, histologic grade, and hormone receptor status remain the most useful indicators of prognosis and therapeutic response. A major obstacle to the translation of research advances into clinically useful prognostic and predictive markers has been the considerable methodologic variability used in the evaluation of the newer markers. It is now generally accepted that, to be useful in patient management, a putative prognostic or predictive marker must have clinical importance, independence, significance, and standardization with regard to methods, interpretation, and reporting. It is hoped that recognition and adoption of these criteria will serve to clarify the value of newer biologic and molecular markers. [J Natl Cancer Inst Monogr 2001;30:22-6]

The identification of new biologic and molecular indicators of clinical outcome and response to therapy in patients with breast cancer has been an area of active investigation during the last two decades. Unfortunately, studies of these new markers have often yielded contradictory results and clinical confusion. Furthermore, even the results of studies of the same marker are often difficult to compare because of differences in treatment, study design, patient selection, methodology, and statistical analysis. In fact, despite intensive efforts to identify new prognostic and predictive factors, traditional pathologic factors such as lymph node status, tumor size, histologic type, histologic grade, and hormone receptor status remain the most useful indicators of prognosis in patients with breast cancer (1-6).

LYMPH NODE STATUS

Uniform agreement exists that the status of the axillary lymph nodes is the single most important prognostic factor for patients with breast cancer and that disease-free survival and overall survival decrease as the number of positive lymph nodes increases. Nevertheless, a number of important issues regarding axillary lymph node evaluation need to be addressed. First, methods for the pathologic examination of these lymph nodes are not standardized. For example, although some pathologists submit grossly uninvolved lymph nodes in their entirety for histologic examination, others subject only a single section from such lymph nodes to microscopic scrutiny (6). This difference in method could result in the misclassification of some lymph node-positive patients as lymph node negative. In addition, although sentinel lymph node biopsy is now widely used to evaluate the status of the axilla, methods for examination of sentinel lymph nodes are also highly variable. Another unanswered clinical question concerns the significance of axillary lymph node micrometastases, particularly those identified exclusively by the use of immunohistochemistry. Approximately 10%-20% of patients considered to be lymph node negative by conventional

examination of the axillary lymph nodes are found to have identifiable tumor cells in these lymph nodes when these lymph nodes are examined by serial sectioning, by immunohistochemical staining, or by both methods. Studies that have sought to evaluate the significance of axillary micrometastases have differed with regard to patient population, treatment, methods to detect tumor cells, and length of follow-up. Virtually all studies with more than 100 patients have shown that the presence of micrometastases detected by serial sectioning, immunohistochemistry, or both methods is associated with a small but significant decrease in disease-free survival, overall survival, or both (7,8). However, most of these studies have been retrospective and were not initially designed to address this question. Furthermore, in some of these studies, it is not clear whether the prognostic significance of micrometastases is independent of other factors such as tumor size or lymphatic vessel invasion. The clinical significance of axillary lymph node micrometastases detected by immunohistochemistry is currently being evaluated in a number of randomized clinical trials, and it is likely that these trials will provide important information about this issue. Until then, most experts agree that it is premature to recommend the routine use of immunohistochemistry to evaluate either sentinel or nonsentinel lymph nodes (9).

TUMOR SIZE

After lymph node status, tumor size is the most important prognostic factor for patients with breast cancer. Even among patients with breast cancers 1 cm and smaller (T1a and T1b), size represents an important prognostic factor for axillary lymph node involvement and outcome (10). It should be noted, however, that the manner in which the pathologic tumor size is reported has not yet been standardized. Some pathologists report the size of the macroscopically identified tumor, some report a microscopic size that includes both the invasive and *in situ* components, and still others report the microscopic size of the invasive component only. Prior studies have shown that, particularly for small breast cancers, a poor correlation often exists between the tumor size determined by gross pathologic examination and the size of the tumor's invasive component as determined by measurement from the histologic sections (11). Moreover, some studies suggest that the size of the invasive component is the most clinically significant determinant of outcome. This was recently recognized in the fifth edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (12), which states that "the pathologic tumor size for classification (T) is a measurement of only the invasive component." Therefore, in the case of a discrepancy between the gross tumor size and the

Affiliations of author: Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA.

Correspondence to: Stuart J. Schnitt, M.D., Department of Pathology, Beth Israel Deaconess Medical Center, East Campus, 330 Brookline Ave., Boston, MA 02215 (e-mail: sschnitt@caregroup.harvard.edu).

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microscopic size of the invasive component of small breast cancers, the microscopic size should take precedence and the microscopic size of the invasive component of the tumor should be indicated in the pathology report and used for pathologic staging.

HISTOLOGIC TYPE

Some histologic types of breast cancer are associated with a particularly favorable clinical outcome (*13,14*). Special-type tumors that consistently have been shown to have an excellent prognosis include tubular, mucinous, adenoid cystic, and invasive cribriform carcinomas. Some authors also place tubulolobular carcinomas and papillary carcinomas in this group. Moreover, Rosen et al. (*14*) have shown that the 20-year recurrence-free survival of special-type tumors 1.1–3.0 cm in size is similar to that of invasive ductal carcinomas both 1 cm and smaller (87% and 86%, respectively). However, strict diagnostic criteria must be employed in order to observe the favorable outcome reported for these lesions.

HISTOLOGIC GRADE

The importance of tumor grading as a prognostic factor in patients with breast cancer has been clearly demonstrated in numerous clinical outcome studies. These studies (*1–6,15–24*) have repeatedly shown higher rates of distant metastasis and poorer survival in patients with higher grade (poorly differentiated) tumors, independent of lymph node status and tumor size. In fact, tumor grading has been shown to be of prognostic value even in patients with breast cancers 1 cm and smaller (*10*). Although a variety of nuclear and histologic grading methods have been used in these studies, the grading method used most often at present is the histologic grading system of Elston and Ellis (*21*). These authors advocate the use of histologic grading for all types of invasive breast cancer; they acknowledge, however, that histologic grade partially defines some of these histologic types (e.g., tubular carcinomas are by definition grade 1 and medullary carcinomas are grade 3 lesions). However, these authors have also pointed out that the combination of histologic type and grade provides a more accurate assessment of prognosis than does histologic type alone (*25*).

Some studies have suggested that histologic grade may provide useful information with regard to response to chemotherapy and may, therefore, be of value as a predictive factor in addition to its role as a prognostic indicator. The results of several studies suggest that the presence of high histologic grade is associated with a better response to certain chemotherapy regimens than is the presence of low histologic grade (*26,27*). However, additional studies are required to define more clearly the relationship between histologic grade and response to chemotherapy.

A frequent criticism of the use of histologic grading is that this assessment is subjective and, as a consequence, prone to considerable interobserver variability (*28–30*). Most of the studies that have suggested this used grading systems lacking precisely defined criteria or did not attempt to educate the participating pathologists in the use of the system evaluated. Recent studies have indicated that the use of strict criteria and guidelines for histologic grading can result in acceptable levels of interobserver agreement and also identify areas that might benefit from refinement. In one of these studies (*31*), six pathologists each graded 75 invasive ductal carcinomas using the Elston and Ellis grading system. Moderate to substantial agreement was found

for the overall histologic grade. There was substantial agreement with regard to tubule formation, moderate agreement with regard to mitotic count, and near moderate agreement for nuclear pleomorphism as determined by generalized κ statistics. The authors (*31*) concluded that this grading system is suitable for use in clinical practice and suggested that efforts to improve agreement on nuclear grading would be of value in further fostering agreement in histologic grading. In another study (*32*), a substantial level of agreement (κ statistic, 0.70) was found among 25 pathologists who used the Elston and Ellis grading system, albeit in a small number of cases.

LYMPHATIC VESSEL INVASION

Lymphatic vessel invasion has been shown in numerous studies to be an important and independent prognostic factor. Its major clinical value at this time is as an aid in identifying lymph node-negative patients at increased risk for axillary lymph node involvement (*34–41*) and adverse outcome (*19,36,37,42,43*). The identification of lymphatic vessel invasion may be of particular importance in patients with T1, lymph node-negative breast cancers, since this finding may permit the identification of a subset of patients at increased risk for axillary lymph node involvement and distant metastasis. For example, in one study (*10*), lymphatic vessel invasion was the only clinical or pathologic factor associated with lymph node metastasis in patients with tumors 1 cm and smaller (T1a and T1b). In that study (*10*), lymph node involvement was present in four of seven patients whose tumors showed lymphatic vessel invasion (57%), compared with only one of 100 patients without lymphatic vessel invasion. In another study of 461 patients with T1, lymph node-negative breast cancer (*14*), patients with tumors lacking lymphatic vessel invasion had a 20-year overall survival rate of 81%, compared with a 64% overall survival rate for those whose tumors exhibited lymphatic vessel invasion. Similar findings have been reported by others (*43–46*), even when the analysis was restricted to the subset of T1 breast cancers that were 1 cm and smaller (*43,44*).

As with histologic grade, the ability of pathologists to reproducibly identify lymphatic vessel invasion has been challenged. For example, in one study (*47*), three pathologists concurred on the presence or absence of lymphatic vessel invasion in only 12 of 35 cases. However, a higher level of interobserver agreement has been noted in other studies (*34–37,46*). In one of these studies in which stringent criteria were employed (*34*), an 85% level of overall agreement between two pathologists was found for the presence or absence of lymphatic vessel invasion. The use of strict criteria for the identification of lymphatic vessel invasion is, therefore, imperative.

HORMONE RECEPTOR STATUS

The presence of steroid hormone receptors (estrogen receptor [ER] and progesterone receptor [PR]) represents a relatively weak prognostic factor for patients with breast cancer, but these receptors are the strongest predictive factors for response to hormonal therapy (*1–6*). In recent years, immunohistochemical staining has replaced the ligand-binding biochemical assay for assessment of ER and PR status. In fact, the immunohistochemical method is easier to perform and has been shown to be equal to or better than the biochemical assay in predicting the response to adjuvant endocrine therapy (*48*). However, issues related to

the standardization of methodology, interpretation, and reporting remain to be resolved (6).

NEWER FACTORS

Numerous biologic and molecular markers have been reported to have prognostic or predictive value (or both) in patients with breast cancer. These markers include apoptosis, oncogenes, suppressor genes, proteases, adhesion molecules, angiogenesis, proliferative rate, and DNA content (ploidy) (1-3,6). Some of the factors initially reported to be significant independent prognostic markers subsequently have been shown to have little or no independent prognostic value (e.g., ploidy and cathepsin D). Among most of the other reported factors, variations in study design, methodology, and statistical analysis have led to conflicting and contradictory data (49). A comprehensive review of biologic and molecular prognostic and predictive factors is beyond the scope of this presentation. However, a few of these factors merit specific comment.

HER2/neu

HER2/neu is reviewed in references (50,51). In 1987, Slamon et al. (52) first reported that amplification of the HER2 gene was an important prognostic factor for patients with breast cancer. Since this initial report, many studies have attempted to assess the prognostic significance and predictive value of HER2 gene amplification or protein overexpression. The role of HER2 as a predictive factor is discussed elsewhere. With regard to HER2 as a prognostic factor, amplification or overexpression (or both) of this gene has been associated with an adverse clinical outcome in most studies of lymph node-positive patients. Its role as an independent prognostic marker in lymph node-negative patients, however, remains an unresolved issue. Substantial methodologic variability has made it difficult to reconcile the results of these studies.

p53

Mutations in the p53 tumor suppressor gene or an accumulation of p53 protein has been reported in approximately 20%-50% of human breast cancers. These phenomena are more often seen in patients with familial/hereditary breast cancer syndromes (much as the familial breast and ovarian cancer and Li-Fraumeni syndromes) than in those with sporadic breast cancer. Recent immunohistochemical studies suggest that p53 protein accumulation is associated with several other adverse prognostic factors such as high tumor grade, high proliferation rate, and ER and PR negativity. The results of several follow-up studies (1,53-57) suggest that p53 may be an independent predictor of decreased disease-free and overall survival in both lymph node-positive and lymph node-negative patients. However, not all studies have found p53 expression to be a significant, independent prognostic factor (1,58,59). Furthermore, not all cases in which p53 expression is detected by immunohistochemistry show p53 mutations (60). Aside from their potential value as a prognostic factor, p53 mutations may be associated with drug or radiation resistance, or both, and may, therefore, be a predictive factor as well (61).

ANGIOGENESIS

A number of studies (1,62-65) have reported an association between the density of microvessels in the tumor stroma (as

detected by immunohistochemical stains for endothelial cells, such as factor VIII-related antigen, CD34, or CD31) and prognosis in patients with breast cancer. These studies have indicated that tumors with numerous microvessels are associated with a poor prognosis. Although these studies also have shown that high microvessel density is associated with larger tumor size, poor tumor differentiation, and lymph node-positive status, the prognostic information provided by microvessel density appears to be independent of these other factors. However, a significant association between high microvessel density and poor prognosis has not been observed in all studies evaluating this relationship (66,67). Regardless of its potential role as a prognostic factor, assessment of microvessel density may ultimately be a useful marker for tumors that might respond to anti-angiogenic therapy (1).

CURRENT STATUS OF PATHOLOGIC FACTORS

Tumor size, histologic type, nuclear grade, proliferative rate, and hormone receptors were considered to be the major useful prognostic factors in patients with lymph node-negative breast cancer at the 1990 National Institutes of Health Consensus Development Conference on the Treatment of Early-Stage Breast Cancer (4). At the 1998 St. Gallen Conference on Adjuvant Therapy of Primary Breast Cancer, lymph node status was recognized as the most important breast cancer prognostic factor. Among patients with lymph node-negative disease, tumor size, histologic or nuclear grade, hormone receptor status, and lymphatic vessel invasion were considered to be the most relevant prognostic factors (5). Most recently, at a 1999 Consensus Conference held under the auspices of the College of American Pathologists (CAP) (6), a multidisciplinary group of pathologists, clinicians, and statisticians reviewed prognostic and predictive factors of breast cancer and categorized them into three groups based on the strength of the published data:

Category I: Well supported by the literature. Generally used in patient management (size, lymph node status, histologic type, histologic grade, mitotic figure count, and hormone receptor status).

Category II: Extensively studied biologically, clinically, or both; tested in clinical trials: Biological and correlative studies were done, as were a few clinical outcome studies (HER2, p53, lymphatic vessel invasion, and other proliferation markers such as MIB-1).

Category III: Currently does not meet criteria for category I or category II (ploidy, cathepsin D, angiogenesis, and others).

Of note, the factors considered most relevant at the 1999 CAP Consensus Conference (category I) are virtually identical to those considered most important at the 1990 National Institutes of Health Consensus Development Conference. The fact that little has changed in this area during the last 10 years serves to emphasize two points: 1) that traditional prognostic and predictive pathologic factors are clinically valuable; and 2) that, despite dramatic advances in our understanding of the molecular biology of breast cancer during the last decade, it is difficult to translate research advances into prognostic and predictive markers that are useful in clinical management. In large part, this is a result of the considerable methodologic variability used in evaluating these newer factors.

The AJCC and the International Union Against Cancer have developed criteria to assess the value of putative prognostic and predictive factors (49). The criteria include clinical importance

(the factor is a powerful predictor that can be used in patient management), independence (the factor retains its prognostic or predictive value when other factors are combined with it), and significance (the prognostic or predictive accuracy of the factor rarely occurs by chance). In addition, there must be standardization with regard to methods, interpretation, and reporting. It is hoped that the recognition and widespread adoption of these criteria will result in greater clarity of the value of the newer putative prognostic and predictive factors.

Finally, although the evaluation of individual prognostic and predictive factors has value, a pressing clinical need exists to develop a comprehensive profile of the biologic and molecular characteristics of tumors that may aid in the assessment of prognosis and the prediction of response to various therapeutic modalities. The tools of modern molecular biology, such as microarray technology, may ultimately provide such an assessment by permitting high throughput and parallel analysis of hundreds or thousands of parameters (68).

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NOTE

Dr. Schnitt is a member of the Genentech HER2 Advisory Board.

Prognostic and Predictive Role of Proliferation Indices in Adjuvant Therapy of Breast Cancer

Maria Grazia Daidone, Rosella Silvestrini

In breast cancer, proliferative activity represents one of the biologic processes most thoroughly investigated for its association with tumor progression. In addition to the mitotic activity component of pathologic grading systems, several proliferation indices have provided independent information on prognosis and response to specific treatments in large retrospective studies. Recently, results from treatment protocols prospectively planned to test the clinical utility of proliferative activity have indicated that tumor cell proliferation markers identify two subsets among patients with lymph node-negative cancers: 1) those at a very low risk of relapse and 2) those who will benefit from regimens including anti-metabolites. Future efforts should compare the prognostic accuracy of different proliferation markers, confirm preliminary evidence of a relationship between proliferation and response to specific systemic treatments, and standardize assay techniques to facilitate their transfer to general oncology practice. [J Natl Cancer Inst Monogr 2001;30:27-35]

BACKGROUND

Tumor-proliferative activity represents one of the cellular functions most thoroughly investigated in breast cancer for its association with neoplastic progression and metastatic potential. Several approaches, in addition to the measure of mitotic activity (volume/corrected mitotic index, mitotic activity index, and mitotic index) used by all pathologic grading systems (1), have been used by pathologists and cell biologists to determine and quantify the whole proliferative fraction or the discrete fractions of cells in specific cell cycle phases on consecutive series of clinical tumors (2,3). Initially, quantitative measurement of cells in the S phase of the cell cycle involved the evaluation of the fraction of tumor cells actively incorporating DNA precursors (labeled pyrimidine bases, such as [³H]thymidine, or halogenated analogues, such as bromodeoxyuridine or iododeoxyuridine) (4). Newer technologies include the evaluation by flow cytometry or image cytometry of cells with a DNA-synthesizing content (5) and the quantitation of the entire fraction of proliferating cells (i.e., the growth fraction) for the availability of antibodies raised against the nuclear antigen Ki-67 expressed by cycling cells (Ki-67/MIB-1, Ki-S2, and Ki-S5) (6,7). Such approaches, which are applied on different types of specimens (viable, frozen, or paraffin-embedded tissues), use different methods of evaluation (autoradiography, immunocytochemistry, or cytometry). Each of these methods has inherent advantages and disadvantages, including different feasibility rates, which for some indices ([³H]thymidine-labeling or bromodeoxyuridine-labeling indices [TLI or BrdULI, respectively]) appear to depend strictly on the availability of fresh tumor tissue, whereas for others (flow cytometric S-phase fraction [SPF]) depend on data analysis techniques and interpretation as well. Moreover,

the different measures of proliferation do not always correlate well with one another in terms of biologic or clinical significance when analyzed on the same case series. In fact, a moderate or poor concordance has been observed not only between proliferation indices detecting cells in different cell cycle phases (i.e., SPF and Ki-67 or MIB-1) but also between indices evaluating the fraction of cells in the same cell cycle phase (i.e., SPF and TLI). In addition, proliferation markers showed slightly different sensitivity and specificity rates when related to clinical outcome (8-32) (Table 1). However, at present, only one study (12) has specifically tested the prognostic capability of several different proliferation indices (BrdULI, MIB-1, and mitotic index) on the same series of lymph node-negative and lymph node-positive breast cancers. The results of this study, however, are not definitive, because a relatively small number of events limited the statistical power in subsets homogeneous for stage or clinical treatment.

PROGNOSTIC ROLE OF PROLIFERATION INDICES

A computerized literature search was performed using PubMed. "Breast cancer" and the name of each of the proliferation indices were selected as keywords. All available articles published as late as July 2000 were included. Original English articles were analyzed and selected for inclusion when they reported data on the relation between proliferation indices and clinical outcome, evaluated via univariate and/or multivariate analyses on independent case series of at least 100 patients with a minimum follow-up of 4 years. A total of 120 papers were identified in which results were reported from studies for which tumor specimens were available for determining proliferation indices without any *a priori* study design or prospective definition of specimen collection procedures at the time of therapeutic trial planning [studies providing level of evidence (LOE) III according to the Tumor Marker Grading Utility System proposed by Hayes et al. (33)]. About one third of those 120 papers dealt with studies carried out on patients with lymph node-negative breast cancers or with tumors at any stage, but for which only local-regional treatment was given. This group of studies analyzed the prognostic role of cell proliferation, i.e., its relation with disease-, event-, or relapse-free survival, in the absence of adjuvant systemic treatment. Regardless of the proliferative marker investigated and the criteria used to classify tumors as slowly or rapidly proliferating (mean, median, or continuous values), high proliferation indices were associated in univariate analyses with a high probability of relapse and death.

Affiliation of authors: Department of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy.

Correspondence to: Maria Grazia Daidone, Ph.D., Department of Experimental Oncology, Unit 10, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian, 1, 20133 Milan, Italy (e-mail: daidone@istitutotumori.mi.it).

See "Note" following "References."

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Table 1. Prognostic comparison of proliferation indices* in breast cancer

Authors (reference No.)	Stage†	No. of cases	Prognostic role on relapse-free survival‡ (univariate/multivariate analysis) for			
			TLI-BrdULI	SPF	Mitotic count	Ki-67-MIB-1
Silvestrini et al. (8)	N-	340	Yes/yes	No/no		
He et al. (9)	Mixed	132	Yes	Yes		
Rudas et al. (10)	Mixed	184	Yes/yes			No/no
Gaglia et al. (11)	Mixed	385	No/no			Yes/yes
Thor et al. (12)	Mixed	394	Yes/no		Yes/yes	Yes/no
Meyer and Province (13)	Mixed	550	Yes/no	Yes/no		
Peiró et al. (14)§	N-	115		Yes/yes	No/no	
Winchester et al. (15)	N-	198		Yes/yes	No/no	
Simpson et al. (16)	N+	257		Yes/no	Yes/yes	
Hatschek et al. (17)	Mixed	421		Yes/yes	Yes/no	
Eskelinen et al. (18)	Mixed	148		Yes/yes	Yes/no	
Lipponen et al. (19)	Mixed	363		Yes/no	Yes/yes	
Joensuu et al. (20)	Mixed	220		Yes/no	Yes/yes	
Keshgegian and Cnaan (21)	Mixed	135		Yes	Yes	Yes
Dettmar et al. (22)	N-	90		Yes/yes		Yes/no
Harbeck et al. (23)	N-	100		Yes/no		Yes/no
Raijlo et al. (24)	N-	212		No/no		Yes/yes
Brown et al. (25)	N-	314		Yes/no		Yes/yes
Wiesener et al. (26)	Mixed	188		No		Yes
Gasparini et al. (27)	Mixed	195		Yes/yes		No/no
Jansen et al. (28)	Mixed	220		Yes/no		Yes/yes
Leong et al. (29)	N-, G1	148			No	No
Clahsen et al. (30)	N-	441			Yes	Yes
Jacquemier et al. (31)	Mixed	162			Yes	Yes
Pietiläinen et al. (32)	Mixed	191			Yes/no	Yes/yes

*TLI = [³H]thymidine-labeling index; BrdULI = bromodeoxyuridine-labeling index; SPF = flow-cytometric S-phase fraction.

†N- = node-negative; N+ = node-positive; G1 = grade 1.

‡In some studies, also or only on overall survival.

§SPF by image analysis.

This finding generally persisted in multivariate analyses in which features related to the patient (age and menopausal status), the disease (tumor size, regional lymph node status, and histologic/cytologic findings), or the biology of the tumor (markers associated with differentiation, hormone responsiveness, neoangiogenesis, and genomic alterations) were also considered (13-15,18,19,23,25,34-63) (Table 2). In particular, proliferation indices such as TLI/BrdULI and SPF maintained their predictivity for disease-, event-, or relapse-free survival and for overall or cancer-specific survival, even in the presence of information provided by histologic or nuclear grade, despite the inclusion of mitotic index in all of the grading systems. Counts of mitotic figures were also independent predictors of relapse or death in the presence of histologic grade (18,52,58,60,61). Outcome studies had previously assessed the prognostic contribution of the different components of grading systems. The independent value of mitotic count clearly emerged in these studies (61,64), suggesting that it is useful to consider it separately, regardless of, or in addition to grade.

The integration of proliferation indices with other clinical and pathobiologic factors has provided a better assessment of risk than the consideration of single variables. TLI was evaluated in a large, single-institution series of lymph node-negative breast cancer patients (38) in the presence of traditional prognostic factors (age, tumor size, estrogen receptor [ER], and progesterone receptor [PgR]). TLI, considered as a continuous variable and categorized by tree-structured regression analysis, was able to define subsets at different risk for local-regional relapse (in association with patient age) and distant metastasis (in association with tumor size and patient age). Cell proliferation alone was an independent prognostic discriminant for intermediate-

size tumors (1-2 cm). Conversely, TLI was not predictive for the occurrence of contralateral cancers. Such findings, originally observed in 1800 cases with a median follow-up of 8 years (38), were recently updated at a follow-up of 10 years in 2250 cases. For local-regional relapse, a hazard ratio (HR) of 1.8 (95% confidence interval [CI] = 1.3 to 2.4) was found for patients aged less than or equal to 55 years with high TLI tumors versus those with low TLI tumors or who were aged more than 55 years (two-sided *P* value referred to Wald chi square = .0001). For distant metastasis, the HR was 1.9 (95% CI = 1.5 to 2.5; two-sided *P* value referred to Wald chi square = .0001) for patients with a tumor size greater than 2 cm and aged 46-65 years or with tumor size 1-2 cm and high TLI and 3.0 (95% CI = 2.3 to 4.0; two-sided *P* value referred to Wald chi square = .0001) for patients with a tumor size greater than 2 cm and aged less than or equal to 45 or 56-65 years when these subgroups were compared with patients at low risk (tumor size ≤ 1 cm or tumor size 1-2 cm and low TLI).

Taken together, findings from phase I and II exploratory studies (65) indicate that proliferation indices are independent predictors of clinical outcome. However, similar to other pathobiologic markers such as tumor size and ER status, the predictive value of proliferation indices tends to decrease with longer follow-up in most series (66,67).

CONTRIBUTION OF PROLIFERATION INDICES TO THE IDENTIFICATION OF SUBSETS OF PATIENTS AT A VERY LOW RISK OF RELAPSE

Recently, the determination of proliferation indices, along with other biomarkers, has been prospectively planned as part of

Table 2. Studies including multivariate analyses of proliferation indices* with other prognostic factors in patients with early breast cancer mainly treated with local-regional therapy until relapse

Authors (reference No.)	Stage†	No. of cases	Follow-up, y	Variables included in the model‡					Other factors	P value§	
				Age	Size	Grade	ER/PgR	DFS		OS	
TLI–BrdULI											
Medri et al. (34)	N–	378	5	•	•	•	•/•	MVD		.08	
Paradiso et al. (35)	N–	101	5	•	•	•	•/•			.04	
Silvestrini et al. (36)	N–	215	5	•	•		•/			.004	.035
Courdi et al. (37)	N–	167	8	•	•	•	•/•			.037	
Silvestrini et al. (38)	N–	1800	8	•	•		•/•			.006	.014
Meyer and Province (13)	N–	414	10	•	•		•/	Nuclear size		NS	.095
Cooke et al. (39)	Mixed	164	8		•			N, HER2/neu			NS
Tubiana et al. (40)	Mixed	125	15		•	•				<.05	.05
SPF											
Sigurdsson et al. (42)	N–	250	4	•	•		•/•	Ploidy		.03	.0001
Joensuu and Toikkanen (52)	N–	123	5		•	•	•/•	Nuclear pleomorphism, necrosis, mitotic count			.003
O'Reilly et al. (43)	N–	169	5		•	•	•/	Ploidy		.05	.045
Brown et al. (25)	N–	314	6	•	•		•/•	Cell type, surgery, treatment, Ki-67, ploidy		.006	
Harbeck et al. (44)	N–	125	6.5		•	•	•/•	uPA, PAI-1, cathepsin D, p53, HER2/neu		NS	
Merkel et al. (45)	N–	280	6.5		•	•		Nuclear grade		NS	.05
Winchester et al. (15)	N–	198	6.5	•	•	•	•/	Nuclear grade, MAI, ploidy	0.03		
Balslev et al. (46)	N–	421	7	•	•	•	•/•	Number of examined N		NS	NS
Aubele et al. (41)¶	N–	329	8					Morphometric features, ploidy		NS	
Stal et al. (47)	N–	152	8	•	•		•/			.035	
Isola et al. (48)	N–	289	8.5		•	•	•/	Ploidy, p53, HER2/neu			.0001
Bosari et al. (49)	N–	136	9					Peritumoral lymphatic and vessel invasion, ploidy		NS	
Johnson et al. (50)	N–	100	9		•	•		Ploidy, HER2/neu			.03
Lipponen et al. (19)	N–	180	10		•	•		Histotype, MI, M/V, ploidy			NS
Peirò et al. (14)¶	N–	115	10	•	•	•	•/	Tubule formation, nuclear grade, vascular invasion, necrosis, desmoplasia, inflammatory reaction, MI, ploidy		.03	NS
Witzig et al. (51)	N–	265	12.5	•	•	•	•/	Number of examined N, histotype, nuclear grade, ploidy		.05	NS
Klintonberg et al. (53)	Mixed	210	5.5				•/	N, clinical stage, ploidy		.038	
Amelov et al. (54)	Mixed	158	6		•	•		N, pathologic stage, ploidy		.020	
Stanton et al. (55)	Mixed	201	8	•	•		•/	N, ploidy, HER2/neu			NS
Eskelinen et al. (18)	Mixed	148	9	•	•	•	•/•	N, histotype, nuclear pleomorphism, intraductal growth, treatment, M/V, ploidy		.022	.003
Fisher et al. (56)	Mixed	377	10	•	•	•		Clinical N		.04	.08
Toikkanen et al. (57)	Mixed	223	25	•	•	•		N, histotype, tumor margin, tubule formation, nuclear pleomorphism, necrosis, intraductal growth, MAI			.02
Mitotic figure count											
Joensuu and Toikkanen (52)	N–	161	5		•	•	•/•	Nuclear pleomorphism, necrosis, SPF			<.0001
Laderkarl and Jensen (58)	N–	98	9	•	•	•	•/	Histotype		.03	
Kato et al. (59)	N–	200	10		•	•		Tumor necrosis, lymphatic and blood vessel invasion, p53, HER2/neu, PCNA		NS	NS
Lipponen et al. (19)	N–	180	10		•	•		Histotype, SPF, ploidy			NS
Peirò et al. (14)	N–	115	10	•	•	•	•/	Tubule formation, nuclear grade, vascular invasion, necrosis, desmoplasia, inflammatory reaction, SPF, ploidy		NS	NS
Aaltomaa et al. (60)	N–	294	13	•	•	•		Histotype, tumor margin, tubule formation, necrosis, intraductal growth		.005	NS
Clayton (61)	N–	378	20	•	•	•	•	Lymphatic invasion, skin/muscle invasion, nuclear grade, nucleoli			<.001
Eskelinen et al. (18)	Mixed	216	9	•	•	•	•/•	N, histotype, treatment		.010	NS
Toikkanen et al. (57)	Mixed	223	28	•	•	•		N, histotype, tumor margin, tubule formation, nuclear pleomorphism, necrosis, intraductal growth, SPF, ploidy			NS

(Table continues)

Table 2 (continued). Studies including multivariate analyses of proliferation indices* with other prognostic factors in patients with early breast cancer mainly treated with local-regional therapy until relapse

Authors (reference No.)	Stage†	No. of cases	Follow-up, y	Variables included in the model‡					P value§	
				Age	Size	Grade	ER/PgR	Other factors	DFS	OS
Ki-67-MIB-1										
Brown et al. (25)	N–	618	6	•	•		•/•	Cell type, surgery, treatment	.0005	
Iacopetta et al. (62)	N–	263	6		•	•	•/•	p53, HER2/neu	NS	NS
Harbeck et al. (23)	N–	100	6.5		•	•	•/•	PAI-1, uPA, cathepsin D, p53, HER2/neu	NS	NS
Pinder et al. (63)	Mixed	177	12		•	•		N	.05	

*TLI = [³H]thymidine-labeling index; BrdULI = bromodeoxyuridine-labeling index; SPF = flow-cytometric S-phase fraction; MAI = mitotic activity index; MI = mitotic index; M/V = volume/corrected mitotic index.

†N- = node-negative.

‡ER = estrogen receptor; PgR = progesterone receptor; MVD = microvessel density; N = axillary nodal status; uPA = urokinase-type plasminogen activator; PAI-1 = inhibitor of uPA; PCNA = proliferating cell nuclear antigen. • = variable included in the model.

§DFS = disease-free survival; OS = overall survival; NS = not significant.

||Diploid tumors.

¶SPF by image cytometry.

adjuvant and neoadjuvant treatment protocols. Although not all studies have been specifically designed to test the predictivity of tumor markers with adequate statistical power, it is likely that they will improve the quality and accuracy of available information. In addition, results are now becoming available from a few valuable prospective studies specifically designed to test marker utility. These studies will provide definitive evaluation of the clinical utility of proliferation indices.

The ability of cell proliferation to identify, in association with traditionally accepted prognostic factors, subgroups at different risk of local-regional or distant relapse has now been confirmed in an LOE II study performed in conjunction with the National Surgical Adjuvant Breast and Bowel Project Protocol B-14 study. Patients with lymph node-negative, ER-positive tumors were randomly assigned to receive adjuvant therapy with tamoxifen or placebo (66). SPF, in association with relatively few other prognostic factors (patient age, PgR status, and tumor size), identified a broad spectrum of risk categories in a subset of more than 800 women in this trial. In fact, the estimated 10-year disease-free survival probability was quite low (<30%) for patients younger than 35 years of age with large, PgR-negative tumors and a very high SPF. Conversely, patients 50 years of age or older with PgR-positive, 1-cm tumors and a negligible proliferative activity had a greater than 80% disease-free survival probability, and intermediate survival values were found for the other combinations of clinical and pathobiologic features. Such a score could provide an accurate assessment of individual patient prognosis and might suggest limiting aggressive adjuvant therapy to only selected women with lymph node-negative, ER-positive tumors.

To test the hypothesis that proliferation markers can discriminate among lymph node-negative patients at different levels of risk, the U.S. Intergroup performed a prospective, randomized clinical trial (LOE I) (68). From 1989 to 1993, 3899 patients with lymph node-negative breast cancer were randomly assigned as follows: Women whose tumors were too small for biochemical ER/PgR assay were classified at a very low risk and received only local-regional treatment. Those with tumors larger than 2 cm or with negative steroid hormone receptors were considered at a high risk, and systemic adjuvant treatment was administered. The "uncertain" risk subset, those patients with tumors less than or equal to 2 cm with positive steroid hormone recep-

tors, had an S-phase fraction measured to discriminate between low and high risk. The 5-year clinical outcome of the subset initially at "uncertain" risk but later classified at low risk because of a low SPF score was superimposable to that of patients initially at low risk because of very small tumor size. The finding validated the utility of cell proliferation and has been independently confirmed in about 700 lymph node-negative tumors in a prospective investigation by Jones et al. (69), in which Ki-67/MIB-1 was considered in addition to SPF.

The prognostic refinement of the intermediate-risk subset by proliferation markers was also studied by our group at the Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy. We investigated whether TLI provides an additive contribution to breast cancer-scoring systems already validated and/or accepted for routine use, based on different clinical and morphopathobiologic features of proven prognostic utility. In lymph node-negative breast cancer, in particular, we tested TLI within risk categories defined according to criteria (based on patient age, tumor size, histologic grade, and ER and PgR status) proposed in the 1998 St. Gallen's International Consensus Conference on the Treatment of Primary Breast Cancer (70). Our study was carried out on a series of 549 women who had primary, resectable invasive breast cancer. All of them were histologically lymph node negative with no radiologic or clinical evidence of distant metastasis, synchronous bilateral tumor, or concomitant second primary neoplasm and underwent surgery at the Istituto Nazionale per lo Studio e la Cura dei Tumori of Milan during the period from January 1991 to December 1994 (median follow-up, 5 years). The case series (Table 3) was consecutive with respect to TLI determined at the time of diagnosis (71) but independent of the series we previously published on TLI (8,36,38). Patients were subjected to mastectomy (132 [24%] cases) or quadrantectomy plus radiotherapy (417 [76%] cases), and all of them underwent axillary lymph node dissection (median number of examined lymph nodes = 18). None of the women received systemic postoperative therapy until new disease manifestation was documented, and all of them underwent follow-up examination at the outpatient clinic of the Istituto Nazionale per lo Studio e la Cura dei Tumori, as previously described (38). Primary treatment failure was defined as the first documented evidence of local recurrence or regional axillary relapse (six events), distant metastasis (53 events), contralateral breast can-

Table 3. Lymph node-negative breast cancer; patient and tumor characteristics*

Characteristic	No. of cases	% of group
Patient age, y		
<35	14	2.6
35-49	174	31.7
50-64	244	44.4
≥65	117	21.3
Tumor size, cm		
≤1	61	11.1
1-2	304	55.4
>2	184	33.5
Histologic grade		
1	72	13.1
2	368	67.0
3	109	19.9
Steroid receptor		
ER-positive	438	79.8
ER-negative	111	20.2
PgR-positive	388	70.7
PgR-negative	161	29.3
TLI, %		
Low	227	41.3
Intermediate	185	33.7
High	137	25.0
Risk categories†		
Low	15	2.7
Intermediate	204	37.2
High	330	60.1

*ER = estrogen receptor; PgR = progesterone receptor; TLI = [³H]thymidine-labeling index.

†According to the 1998 St. Gallen's International Consensus Conference on the Treatment of Primary Breast Cancer (70).

cer (18 events), or a combination of these events. Steroid receptors were evaluated by the dextran-coated charcoal technique (38), and histologic grade was determined according to the procedures of Elston and Ellis (72).

Patient age, tumor size, histologic grade, and TLI (divided into three classes on the basis of its frequency distribution in relapsed cases), but not ER or PgR, provided prognostic information for 5-year relapse (Table 4) and, with the exception of histologic grade, retained their independent relevance even in

multivariate analysis. When age, size, histologic grade, ER, and PgR were combined according to the St. Gallen criteria (70), several risk categories of prognostic importance were defined (Fig. 1). No relapses occurred in the minimal-low-risk subset (ER-positive and/or PgR-positive, grade 1 tumors ≤1 cm in patients older than 35 years [2.7% of the cases]). Relapse rates of 29% and 14%, respectively, were observed for the subsets at high risk (patients <35 years old or with tumors >2 cm, or ER-negative and/or PgR-negative, or grade 3 [60.1% of the cases]) or at intermediate risk (patients not included in the two previous categories, i.e., those with tumors 1-2 cm in size, ER-positive and/or PgR-positive, and of histologic grade 1 or 2 [37.2% of the cases]). A statistically significant association (chi square test; two-sided *P* = .014) was observed between the St. Gallen categories and TLI. The fraction of slowly proliferating tumors decreased from the minimal-low-risk to the high-risk subset (from 60% to 40% of the cases), with a parallel increase in the fraction of rapidly proliferating tumors (from 13% to 30% of the cases). The overall concordance among the three St. Gallen or TLI classes was limited to only about one third of the cases. TLI provided no prognostic additive information within the high-risk subset (5-year relapse probability for high versus low-intermediate TLI, 29% and 28%, respectively), in which at least one of five unfavorable factors was already present. Conversely, in the intermediate-risk group, TLI was able to separate further the patients into two subsets with different relapse probabilities (5-year relapse for the high [30%] versus the low-intermediate [9%] TLI subsets; HR = 3.4, 95% CI = 1.4 to 8.3; two-sided *P* value referred to Wald chi square = .0076) (Fig. 2). The results, in keeping with those previously reported for SPF (68), support the use of cell proliferation for better prognostic resolution within intermediate-risk categories, even in the presence of information provided by grading.

CLINICAL UTILITY OF PROLIFERATION INDICES IN IDENTIFYING HIGH-RISK LYMPH NODE-NEGATIVE PATIENTS WHO NEED AGGRESSIVE TREATMENT

In the last decade, an additional aim of prospective studies using cell kinetic features was to investigate whether lymph

Table 4. Lymph node-negative breast cancer; univariate and multivariate analyses by Cox model of relapse-free survival at 5 years*

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>P</i> value†	HR	95% CI	<i>P</i> value‡
Patient age, y: <35 vs. ≥35‡	3.1	1.38 to 7.18	.0066	2.9	1.27 to 6.79	.0119
Tumor size, cm						
1-2 vs. ≤1‡	1.7	0.70 to 3.90	.244	1.5	0.64 to 3.54	.350
>2 vs. ≤1‡	2.8	1.17 to 6.46	.020	2.4	0.99 to 5.62	.052
Histologic grade						
2 vs. 1‡	1.5	0.72 to 3.14	.275	1.4	0.64 to 2.87	.422
3 vs. 1‡	2.2	0.99 to 4.86	.053	1.6	0.67 to 3.69	.304
Steroid receptor status						
ER-negative vs. ER-positive‡	1.4	0.89 to 2.23	.139	1.1	0.62 to 1.99	.724
PgR-negative vs. PgR-positive‡	1.3	0.83 to 1.93	.267	1.0	0.56 to 1.66	.884
TLI, %						
Intermediate vs. low‡	1.5	0.92 to 2.42	.106	1.5	0.94 to 2.48	.089
High vs. low‡	1.9	1.13 to 3.09	.014	1.9	1.10 to 3.11	.020

*HR = hazard ratio for relapse; CI = confidence interval; ER = estrogen receptor; PgR = progesterone receptor; TLI = [³H]thymidine-labeling index.

†Two-sided *P* values referred to Wald chi-square.

‡Reference category.

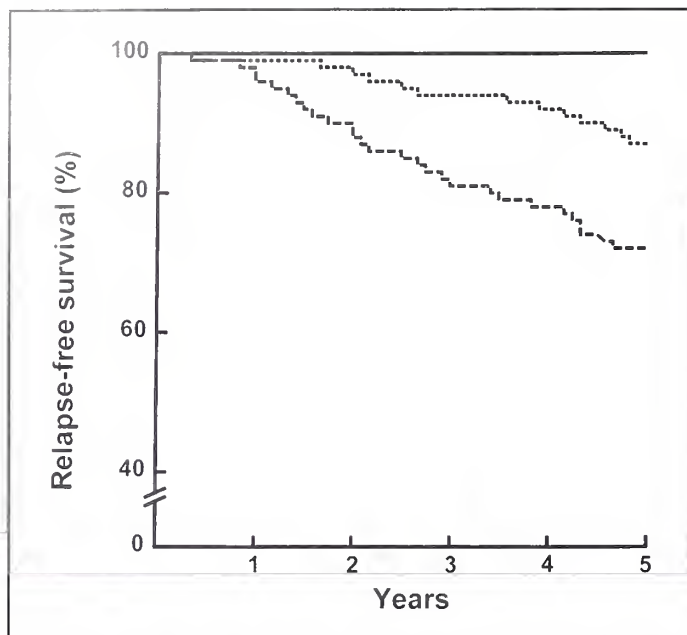


Fig. 1. Relapse-free survival curves for 549 patients with resectable breast cancer by risk categories according to the 1998 St. Gallen's International Consensus Conference on the Treatment of Primary Breast Cancer (70). **Solid line:** minimal-low-risk subset (including estrogen receptor [ER]-positive and/or progesterone receptor [PgR]-positive, grade 1 tumors ≤ 1 cm from patients ≥ 35 years old; 15 cases); **dotted line:** intermediate-risk subset (including ER-positive and/or PgR-positive, grade 1–2, 1- to 2-cm tumors from patients ≥ 35 years old; 204 cases); **dashed line:** high-risk subset (including patients with at least one of the following unfavorable factors: age < 35 years, tumor size > 2 cm, ER-negative or PgR-negative, or grade 3; 330 cases).

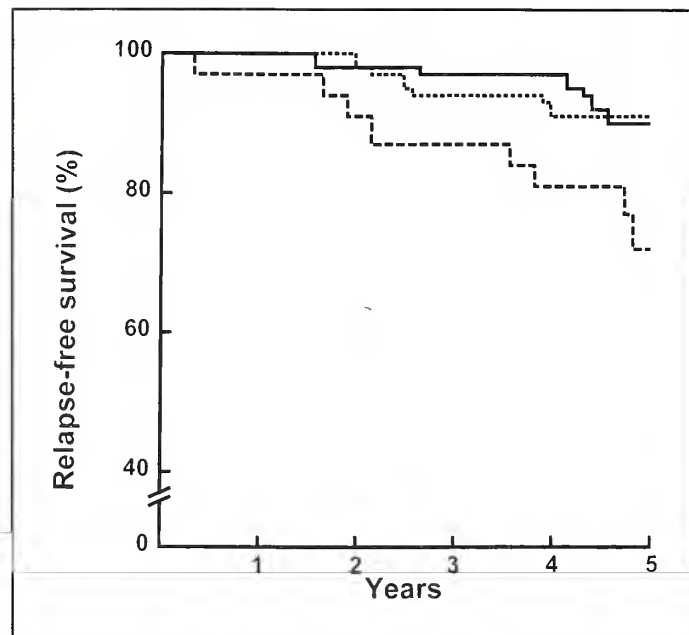


Fig. 2. Relapse-free survival curves for 204 patients with resectable breast cancer classified at an intermediate risk of relapse (with estrogen receptor-positive and/or progesterone receptor-positive, grade 1–2, 1- to 2-cm tumors and aged ≥ 35 years) according to the 1998 St. Gallen's International Consensus Conference on the Treatment of Primary Breast Cancer (70). Relapse-free survival was analyzed as a function of tumor [^3H]thymidine-labeling index (TLI). **Solid line:** low TLI (0.1%–2.4%, 83 cases); **dotted line:** intermediate TLI (2.5%–4.4%, 83 cases); **dashed line:** high TLI ($> 4.4\%$, 38 cases). Intermediate versus low TLI subsets: hazard ratio (HR) = 1.0; 95% confidence intervals (CI) = 0.3 to 3.2; two-sided P value referred to Wald chi square = .972. High versus low-intermediate TLI subsets: HR = 3.4; 95% CI = 1.4 to 8.3; two-sided P value referred to Wald chi square = .0076.

node-negative breast cancer patients defined as high risk on the basis of tumor cell proliferation could benefit from adjuvant polychemotherapy. Three phase III randomized trials using TLI (73,74) or the mitotic activity index (75) have been activated in Europe. Patients were randomly assigned to receive adjuvant chemotherapy (e.g., cyclophosphamide, methotrexate, and 5-fluorouracil [CMF] or 5-fluorouracil, doxorubicin, and cyclophosphamide) versus no systemic therapy. These studies measured tumor cell proliferation and instituted quality-control programs for analytical and preanalytical phases of cell kinetic determinations (71,76–79).

Results are available from the multicenter Italian study by Amadori et al. (73). Patients were eligible for the study if they were younger than 70 years of age, underwent radical or conservative resection plus radiotherapy, had lymph node-negative tumors histologically assessed, and had TLI and ER determinations available. A total of 278 patients with high ($> 3\%$) TLI were accrued and randomly assigned to receive CMF or no further treatment. Disease-free survival probability curves showed a 5-year benefit in CMF-treated patients versus untreated patients (83% versus 72%), with a reduction in local–regional (6.4% versus 2.9%) and distant relapses (21.3% versus 12.4%) and in the annual risk of relapse (approximately 40%), even when adjusted for age, tumor size, type of surgery, and PgR content. The benefit of CMF treatment was mostly evident for cases at very high risk, i.e., with TLI values greater than 6.8% (corresponding to the third tertile of TLI frequency distribution).

The results support the use of cell proliferation to select patients with lymph node-negative tumors at a high risk of recurrence. The finding of a greater benefit from antimetabolite-based

regimens in tumors with the highest proliferation is in keeping with the evidence from studies measuring cell proliferation as part of prospective randomized clinical trials comparing systemic treatment with observation or radiotherapy in either lymph node-positive or high-risk, lymph node-negative patients (80–82).

PROLIFERATION INDICES AND RESPONSE TO SYSTEMIC ADJUVANT TREATMENTS

There has been a renewed emphasis in the search for biologic predictive factors, i.e., markers able to identify patients who are more or less likely to benefit from specific therapies. Compared with prognostic factors, however, this field of research is more difficult to investigate, since the ideal study should include the prospective evaluation of the marker within the context of a randomized clinical study designed to compare systemic therapies with local–regional therapies. Proliferative activity represents a biomarker that may be both prognostic and predictive. As for most putative biologic predictors, present data mainly acquired from LOE III studies are insufficient to draw firm conclusions regarding the predictive role of proliferation indices in choosing either endocrine therapy or chemotherapy and are only suggestive of relations that should be further investigated and analyzed.

As regards predictors of response to chemotherapy, emerging evidence from adjuvant and neoadjuvant studies (83,84) generally indicates a benefit of polychemotherapy including S-phase-

specific drugs for patients with rapidly proliferating tumors, even though such a finding is not unequivocal. In fact, in companion studies of prospective, randomized clinical trials comparing systemic treatment with observation or radiotherapy, an advantage from CMF on long-term outcome was present only in rapidly proliferating tumors (80) or in rapidly and in slowly proliferating tumors (85), but the benefit was greater in the former (81,82).

Up to now, few studies have investigated whether cell kinetics provides information on the efficacy of different treatment schedules. In an ancillary study analyzing 70% of the cases entered in a randomized treatment protocol aimed at comparing alternating and sequential regimens of doxorubicin and CMF in breast cancer patients who had more than three positive axillary lymph nodes, the benefit of sequential administration was mainly evident in patients with tumors with low to intermediate proliferation rates (86). The data could be explained by a partial synchronization of cells in the G₂-M phase of the cell cycle following the initial administration of doxorubicin at a high dose intensity and by a subsequent presentation during the CMF cycles of a large number of cells sensitive to S-phase-specific drugs.

As regards prediction of response to endocrine therapy, evidence from adjuvant and neoadjuvant studies generally indicates a greater benefit for patients with slowly proliferating tumors, either within ER-positive subsets or in the presence of information provided by PgR (87,88), although contrasting results are present in the literature (89). All of the data have been obtained from retrospective clinical analyses, and prospective studies are needed.

CONCLUSIONS

Proliferation indices can be markers of clinical utility. In fact, in lymph node-negative breast cancers, the usefulness of cell proliferation in identifying subsets at a very low risk of relapse has been assessed in large retrospective studies and validated in prospective studies (68,69), and the benefit from chemotherapy regimens including antimetabolites in treating rapidly proliferating tumors has been assessed in a phase III prospective study (73). Further effort should be made to define the relative prognostic accuracy of the different proliferation markers within prospective clinical trials and to confirm the preliminary evidence of a relationship between proliferation and response to specific systemic treatments.

In addition to clinicobiologic effectiveness and usefulness, laboratory quality-control programs should be considered to promote the transferability of these measurements from the research laboratories to general practice (90). Effort should be devoted to standardize methodologies and interpretation criteria to improve reliability, accuracy, and reproducibility of assay results within and among the different laboratories. Moreover, links should be created among the different quality-control programs so as to share common methodologies so that clinical trial results can be extrapolated to routine practice.

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Efficacy of Systemic Adjuvant Therapy for Breast Cancer in African-American and Caucasian Women

James J. Dignam

Observed variations in breast cancer survival by racial/ethnic background have been attributed to many factors, including differences in clinical and pathologic disease features at diagnosis and economic resource inequities that may affect treatment access and quality. In this report, we examine outcomes for African-American and Caucasian breast cancer patients participating in selected randomized clinical trials of the National Surgical Adjuvant Breast and Bowel Project (NSABP) to determine whether prognosis or efficacy of systemic adjuvant therapy differed between these groups. Randomized clinical trials offer the advantages of a similar disease stage and a uniform treatment plan for all participants. Patients from four NSABP trials enrolling patients from 1982 through 1994 with axillary lymph node-negative disease (543 African-American and 7582 Caucasian) and three trials enrolling patients from 1984 through 1991 with axillary lymph node-positive disease (548 African-American and 4986 Caucasian) were included. Disease-free survival (DFS), which was defined as time on study free of breast cancer recurrence, second primary cancer, or death preceding these events, and survival risk ratios (RRs) with two-sided 95% confidence intervals (CIs) for African-Americans versus Caucasians were computed from Cox proportional hazards models that included relevant prognostic covariates. Treatment benefits for the therapies evaluated in these trials were estimated separately for African-Americans and for Caucasians. Among patients with lymph node-negative disease, African-Americans had similar DFS rates to Caucasians (African-American/Caucasian RR = 1.06, 95% CI = 0.92 to 1.23) but had modestly greater mortality rates (RR = 1.21, 95% CI = 1.01 to 1.46). Among lymph node-positive patients, DFS was similar (RR = 1.04, 95% CI = 0.93 to 1.17) and survival was again less favorable for African-Americans (RR = 1.18, 95% CI = 1.03 to 1.34). Survival excluding deaths most likely attributable to causes other than cancer was similar between African-Americans and Caucasians (RR = 1.08 [95% CI = 0.88 to 1.33] for lymph node-negative patients and RR = 1.09 [95% CI = 0.96 to 1.25] for lymph node-positive patients). Among lymph node-negative and lymph node-positive patients, African-Americans and Caucasians realized comparable benefit from either the addition of chemotherapy or tamoxifen to surgery alone or the addition of chemotherapy to tamoxifen. In summary, African-American women and Caucasian women who were diagnosed at a comparable disease stage and were similarly treated tended to experience similar breast cancer prognosis. However, a mortality deficit persisted for African-American women relative to Caucasian women, which may be in part due to greater mortality from noncancer causes among African-Americans. Benefit from systemic adjuvant therapy for recurrence and mortality reduction was comparable between African-Americans and Caucasians. This study and investigations in other health-care settings suggest that Af-

frican-American women and Caucasian women with breast cancer derive a similar benefit from systemic adjuvant therapy when it is administered in accordance with their clinical and pathologic disease presentation. [J Natl Cancer Inst Monogr 2001;30:36-43]

Differences in breast cancer survival among racial/ethnic groups have been noted in many studies, as well as in national cancer statistics summaries (1,2). Numerous factors have been implicated as sources of these differences, including disease characteristics at diagnosis, economic resource inequities and other social factors, and disparities in treatment access and (possibly) efficacy. Asian-Americans tend to have lower incidence of breast cancer than do Caucasians, and they also have a superior prognosis, in part because of earlier stage at diagnosis and favorable disease features (3,4). Women of Hispanic origin are more frequently diagnosed at a later stage and more often exhibit other less favorable disease features relative to non-Hispanic Caucasians (3,5-7). American Indian women more often are diagnosed at a later stage and have poorer survival rates than do both Hispanics and Caucasians (7,8). The most extensively investigated disparity in breast cancer prognosis is that between African-Americans and Caucasians. There is general consensus that African-American women are more often diagnosed at a later stage, have larger tumors with less favorable characteristics, and may suffer barriers to quality care, resulting in poorer survival [for review, see (9)].

The existence of cancer outcome disparities between race groups despite improvements in diagnosis and treatment over recent decades is considered by most to be largely a consequence of personal and institutional resource limitations in specific racial/ethnic communities rather than of any intrinsic aspect of race itself, although cultural and social factors associated with ethnicity may also play a role (10-14) [for review, see (15)]. In circumstances where resource disparities are absent, cancer-screening participation, disease stage at diagnosis, treatment, and subsequent outcomes are similar regardless of race, suggesting economic inequity as a major explanatory factor in outcome disparities (16-21).

To evaluate the role of disease stage at diagnosis and subsequent treatment in reducing outcome disparities among patients of different race backgrounds, we examined breast cancer prognosis among African-American and Caucasian women participating in randomized clinical trials of the National Surgical Ad-

Affiliations of author: Biostatistical Center, National Surgical Adjuvant Breast and Bowel Project, Pittsburgh, PA, and Department of Health Studies, University of Chicago, IL.

Correspondence to: James J. Dignam, Ph.D., Department of Health Studies, 5841 South Maryland Ave., MC 2007, University of Chicago, Chicago, IL 60637 (e-mail: jdignam@health.bsd.uchicago.edu).

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juvant Breast and Bowel Project (NSABP). The randomized clinical trials provide a setting for a comparison of outcomes among women diagnosed at the same disease stage and treated uniformly according to a prescribed protocol. In this study, we address 1) whether outcomes among African-American and Caucasian women included in these trials are more similar than those observed in the population at large and whether specific demographic, clinical, and biologic factors might explain any differences observed and 2) whether there is evidence of differential efficacy of systemic adjuvant therapy between African-Americans and Caucasians. Previous studies from the NSABP and others have found outcomes among Caucasians and African-Americans to be more comparable in clinical trials and other equal-care settings than those that have been observed in population-based studies (9,21–26).

PATIENTS AND METHODS

Trials and Patients Included

The NSABP is a National Cancer Institute-sponsored cooperative clinical trials group evaluating treatments for breast and colorectal cancers. The group is headquartered in Pittsburgh, PA, and participating institutions enroll patients throughout North America. Beginning in the early 1980s, a series of trials were conducted among patients with operable breast cancer and axillary lymph nodes that were pathologically negative for tumor cells. In 1981, NSABP Protocol B-13 was opened for patients with lymph node-negative disease and estrogen receptor (ER)-negative tumors (<10 fmol/mg cytosol protein) and compared surgery alone with surgery followed by sequential methotrexate and 5-fluorouracil (M→F). Concurrently, NSABP Protocol B-14 enrolled lymph node-negative women with ER-positive breast tumors (≥ 10 fmol) and compared long-term tamoxifen (5 years) with placebo after surgery. Following results from these trials indicating an advantage for active treatment, two trials for this same class of patients were initiated in 1988: Protocol B-19 compared M→F with conventional cyclophosphamide (C), methotrexate (M), and 5-fluorouracil (F) (CMF) among those patients with ER-negative tumors. Protocol B-20 compared the addition of M→F or CMF with tamoxifen among patients with ER-positive tumors.

During the 1980s, continuing developments in the treatment of patients with axillary lymph node-positive breast cancer led to several trials evaluating the utility of the doxorubicin (Adriamycin)–cyclophosphamide (AC) chemotherapy regimen. From 1984 through 1989, NSABP Protocols B-15 and B-16 accrued patients with operable breast tumors and positive axillary lymph nodes. Eligible patients for Protocol B-15 were those under 50 years of age and those aged 50–59 years with progesterone receptor (PgR)-negative tumors (<10 fmol). These patients were randomly assigned to receive conventional CMF, AC for four courses, or AC followed by CMF. Protocol B-16 enrolled patients aged 50–59 years with PgR-positive tumors and all patients aged 60–70 years. These patients were randomly assigned to receive either tamoxifen alone or tamoxifen and AC (additional treatment arms using L-phenylalanine mustard (L-PAM) were included in comparisons of race but omitted from evaluation of treatment efficacy in African-Americans and Caucasians, since this agent is no longer in use). Protocol B-22, which accrued patients from 1989 through 1991, evaluated increased and intensified doses of C in the AC regimen.

Patients from these trials were excluded from this analysis if they had either no follow-up information or unknown clinical or pathologic tumor size or if their axillary lymph node status did not match the protocol entry criteria for the trial to which the patient was enrolled. The proportion of patients with no follow-up ($<1\%$ of patients in any one trial), missing tumor size, or incorrect lymph node status did not differ significantly by race. From the trials for lymph node-negative breast cancer, 543 African-American, 7582 Caucasian, and 456 patients of other or unknown race were included in this analysis. Among patients included from the trials for lymph node-positive breast cancer, there were 548 African-Americans, 4986 Caucasians, and 317 patients of other or unknown race. Because this latter group is heterogeneous with regard to race, we do not provide specific inference about women in this category. Outcomes and characteristics for these women as a group were similar to those of Caucasians. African-Americans constituted 9%–11% of patients from individual trials in which patients with ER-negative tumors were enrolled (B-13 and B-19), in which younger and PgR-negative patients were enrolled (B-15), or in which ER was not an entry criterion (B-22). In trials that enrolled ER-positive (B-14 and B-20) or predominantly ER-positive (B-16) patients, African-Americans constituted 5%–6% of all patients enrolled, reflecting the lower incidence of ER-positive tumors observed among African-Americans in the population of breast cancer patients (5,27,28).

Further study design details and major findings from these trials have been presented previously (29–34). Results in this study reflect data reported to the NSABP data coordinating center as of December 31, 1999. Follow-up is administratively censored at 12 years for all studies.

Endpoints and Statistical Methods

Frequency distributions for selected characteristics were compared by using chi-square and exact tests. Two-sample *t* tests and appropriate nonparametric counterparts were used to compare means or medians of continuous distributions. Time-dependent endpoints were 1) disease-free survival (DFS) time, defined as time from surgery until breast cancer recurrence, new primary cancer, or death before recurrence or new primary cancer; 2) recurrence-free survival (RFS) time, which is defined as time until breast cancer recurrence, with other events treated as censored observations; and 3) survival time, which is defined as time from surgery until death from any cause.

The Cox proportional hazards model was used to compute risk ratios for African-Americans relative to Caucasians for each of the failure endpoints. Other potentially confounding covariates were included in these models. Evidence of a differential benefit for treatment according to race was tested by including cross-product terms representing the race \times treatment interaction and testing the significance of these terms via likelihood ratio methods. Treatment comparisons were conducted separately for African-Americans and Caucasians irrespective of statistical significance of the interaction to illustrate the direction, magnitude, and variability of the treatment effect estimates within these groups. Analyses of treatment efficacy were confined to the individual trials. Analyses of differences between African-Americans and Caucasians were conducted 1) within individual trials, 2) combined across trials according to ER status, and 3) combined overall. All confidence intervals (CIs) shown are based on a two-sided 0.05 test criterion.

Characteristics of African-Americans and Caucasians With Breast Cancer

Characteristics at diagnosis were compared for African-American and Caucasian patients separately by lymph node and ER status (not shown). Characteristics were somewhat less favorable for African-Americans compared with Caucasians but did not differ as greatly as was reported in population-based studies, since patients were similar with respect to disease stage within each trial. Among patients with lymph node-negative disease, the median age at diagnosis was 1 (ER-negative patients) to 3 (ER-positive patients) years less for African-Americans. African-Americans had tumors that averaged about one-half centimeter larger than those of Caucasians. Among lymph node-positive patients, African-Americans again tended to be 1–3 years younger at diagnosis and had larger tumors than their Caucasian counterparts, while the number of lymph nodes positive for tumor cells did not differ between African-Americans and Caucasians.

Outcomes for African-Americans Relative to Caucasians

Risk ratios (RRs), which represent the failure hazard ratio of African-Americans to Caucasians, taking into account other prognostic covariates (age, tumor size, and treatment), were computed for patients from trials for lymph node-negative patients (Fig. 1). DFS was similar and did not differ significantly in individual trials or overall. ER-negative African-American and Caucasian patients had similar DFS (African-American/Caucasian RR = 1.07; 95% CI = 0.84 to 1.35), as did those with ER positive tumors (RR = 1.05; 95% CI = 0.87 to 1.27). Recurrence-free survival results were similar (not shown). African-American patients consistently showed a modestly increased mortality risk (Fig. 1); ER-negative RR = 1.30 [95% CI = 0.98 to 1.73]; ER-positive RR = 1.17 [95% CI = 0.92 to

1.49]. For all patients combined, there was a 21% excess risk of mortality for African-Americans (RR = 1.21; 95% CI = 1.01 to 1.46).

RRs from the lymph node-positive trials are shown in Fig. 2. DFS was similar for those with both ER-negative (RR = 1.02; 95% CI = 0.88 to 1.18) and ER-positive (RR = 1.09; 95% CI = 0.90 to 1.32) tumors. Results for RFS were similar (not shown). African-American patients again had higher mortality risk (Fig. 2; ER-negative RR = 1.06 [95% CI = 0.90 to 1.25]; ER-positive RR = 1.46 [95% CI = 1.18 to 1.80]) and an 18% excess mortality risk overall (RR = 1.18; 95% CI = 1.03 to 1.34).

Because mortality differences persisted despite similar outcomes with respect to breast cancer recurrence, we compared survival, treating deaths before documented breast cancer recurrence or second primary cancer as censored observations. These deaths are usually attributed to causes other than cancer (although patients with inadequately documented breast cancer recurrence may also be included in this category), and there were marginally more African-American women with these events in every study examined. Mortality outcomes did not differ significantly between African-Americans and Caucasians after these events were censored (RR = 1.08 [95% CI = 0.88 to 1.33] for lymph node-negative patients; RR = 1.09 [95% CI = 0.96 to 1.25] for lymph node-positive patients).

Treatment Efficacy Estimates

The proportional hazards models were used to evaluate whether there was a differential treatment response between African-Americans and Caucasians by adding model terms representing the interaction effect and evaluating these terms via likelihood ratio tests. In no case was a statistically significant interaction between race and treatment found.

To illustrate the potential magnitude of treatment efficacy among African-Americans and Caucasians, treatment compari-

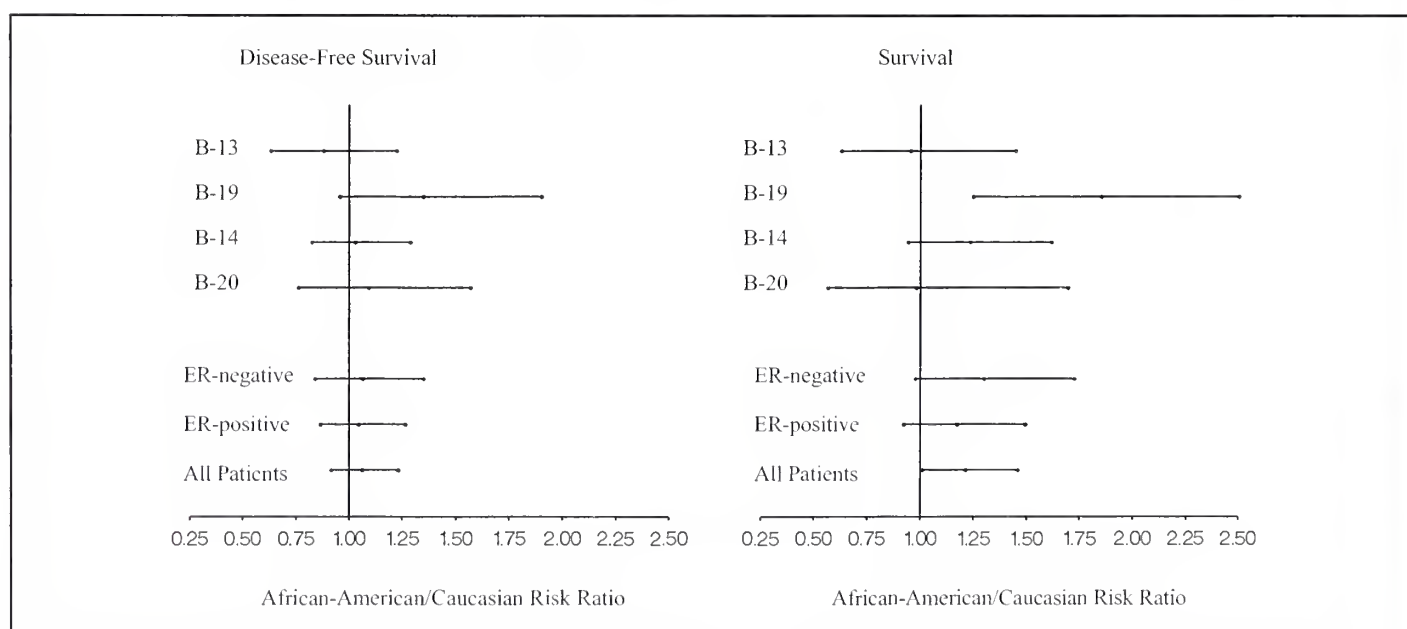


Fig. 1. Risk ratios and 95% confidence intervals for patients ($n = 543$ African-Americans and 7582 Caucasians) from trials for lymph node-negative breast cancer, shown by individual trial, combined over trials according to estrogen receptor (ER) status, and combined overall. African-American/Caucasian risk ratios for disease-free survival (left) and survival (right) are computed from the Cox proportional hazards model including covariates for age at diagnosis, tumor size, and treatment group.

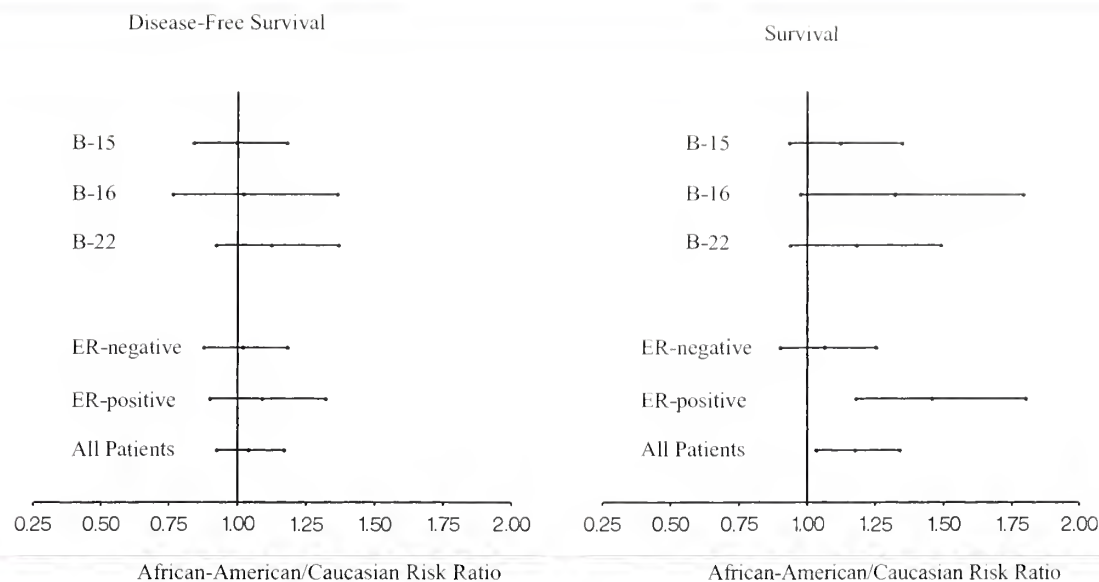


Fig. 2. Risk ratios and 95% confidence intervals for patients (n = 548 African-Americans and 4986 Caucasians) from trials for lymph node-positive breast cancer, shown by individual trial, combined over trials according to estrogen receptor (ER) status, and combined overall. African-American/Caucasian risk ratios for disease-free survival (left) and survival (right) are computed from the Cox proportional hazards model including covariates for age at diagnosis, tumor size, number of positive lymph nodes, and treatment group.

sons were conducted separately in each group. These comparisons were confined to the individual trials (e.g., not among patients aggregated over trials) to avoid temporal or other biases that might be introduced from comparisons of treatment groups from different trials. Note that each of these comparisons is tantamount to a randomized trial comparison within race group, since patients entering the trials are randomly assigned equally irrespective of race and since relevant covariates are balanced by treatment arm. For lymph node-negative patients, the addition of chemotherapy to surgery alone among ER-negative patients (B-13) resulted in an approximate 33% reduction in DFS events (Table 1). The advantage of CMF over the MF regimen among ER-negative patients (B-19) was apparent among Caucasians, while outcomes for African-Americans appeared to be similar with either treatment (a formal interaction test was not significant). For ER-positive patients, the addition of tamoxifen to surgery (B-14) conferred a benefit of roughly a 25%–35% reduction in events for both African-Americans and Caucasians. For the addition of chemotherapy to tamoxifen among ER-

positive patients (B-20), both African-Americans and Caucasians benefited with respect to reduction in DFS events (Table 1). Results for survival were largely similar to those for DFS but with less precision in estimates because of the relatively good survival prognosis among lymph node-negative patients (not shown).
 Treatment efficacy comparisons for lymph node-positive patients were also similar between African-Americans and Caucasians (Table 2). The AC regimen was found to be at least as efficacious as the conventional CMF regimen both overall and among African-Americans or Caucasians (B-15). The addition of AC to tamoxifen in older and largely ER-positive patients (B-16) was beneficial in both African-Americans and Caucasians. Increased and increased/intensified dose of cyclophosphamide in the AC regimen (B-22) did not improve outcomes overall or among Caucasians, and there was no evidence that African-Americans in particular benefited from this regimen (Table 2). Results for survival were similar (not shown).

Table 1. Treatment efficacy among African-Americans and Caucasians in National Surgical Adjuvant Breast and Bowel Project lymph node-negative breast cancer trials*

Trial: treatment comparison* (No. of patients)	Disease-free survival risk ratio (95% confidence interval)		
	African-Americans	Caucasians	All patients
B-13: M → F/surgery alone (107 African-American and 922 Caucasian)	0.68 (0.35 to 1.31)	0.66 (0.53 to 0.82)	0.67 (0.55 to 0.81)
B-19: CMF/M → F (99 African-American and 907 Caucasian)	0.97 (0.50 to 1.89)	0.69 (0.54 to 0.88)	0.70 (0.56 to 0.88)
B-14: tamoxifen/placebo (201 African-American and 3711 Caucasian)	0.75 (0.49 to 1.16)	0.65 (0.59 to 0.73)	0.66 (0.60 to 0.73)
B-20: tamoxifen + M → F or CMF/tamoxifen (136 African-American and 2042 Caucasian)	0.72 (0.35 to 1.48)	0.74 (0.61 to 0.90)	0.74 (0.62 to 0.89)

*Table entries are risk ratios for first treatment/second treatment. M → F = sequential methotrexate and 5-fluorouracil; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil. All patients underwent surgery before adjuvant therapy.

Trial: treatment comparison* (No. of patients)	Disease-free survival risk ratio (95% confidence interval)		
	African-Americans	Caucasians	All patients
B-15: CMF/AC (253 African-American and 1900 Caucasian)	1.08 (0.73 to 1.62)	1.06 (0.92 to 1.23)	1.06 (0.93 to 1.21)
B-16: tamoxifen + AC/tamoxifen (83 African-American and 1127 Caucasian)	0.39 (0.18 to 0.85)	0.84 (0.70 to 1.02)	0.80 (0.67 to 0.96)
B-22: increased C/C in the AC regimen (212 African-American and 1959 Caucasian)	1.34 (0.87 to 2.06)	0.95 (0.83 to 1.08)	0.97 (0.86 to 1.10)

*Table entries are risk ratios for first treatment/second treatment. CMF = cyclophosphamide, methotrexate, and 5-fluorouracil. AC = doxorubicin (Adriamycin) and cyclophosphamide. All patients underwent surgery before adjuvant therapy.

DISCUSSION

A number of studies (21,25,26) have found that, when disease stage and treatment are comparable, breast cancer prognosis for African-Americans and Caucasians is similar. Similarly, when disease stage and important socioeconomic variables, which are highly correlated with care access and quality, are comparable, breast cancer outcomes for African-Americans and Caucasians are similar (17,19). Residual differences noted in many studies (5,27,28,35) may be caused by disease features other than stage, such as lack of hormone receptors and presence of necrosis, that could impart increased recurrence risk for African-American women. Other, yet unidentified factors may also contribute to poorer outcomes for patients of specific racial/ethnic background. Most likely, these would be confined to additional tumor-specific features that may be more prevalent in some race groups, as have been identified previously.

Data presented here from a series of randomized clinical trials conducted during the last 20 years provide a unique opportunity to compare outcomes for patients with similar disease receiving the same care and, furthermore, offer an opportunity to evaluate treatment benefit among African-Americans, albeit with a smaller than desirable sample size, under random treatment assignment—the recognized “gold standard” for therapy development. Some drawbacks of clinical trial databases include both a lack of socioeconomic data that might be relevant to this question and the small number of women of many race groups. Even if proportionally representative sampling is achieved, then an approximate 9 to 1 ratio of Caucasians to African-Americans will be enrolled. Women of other ethnicity backgrounds have even less representation in actual numbers.

Main findings from this recent survey of trials consistently indicated equivalent DFS and RFS outcomes for African-Americans and Caucasians within trials and over studies, combined. However, a mortality deficit for African-American women was consistently seen, with an approximate 21% excess risk of mortality among lymph node-negative African-American patients and a 17% excess risk of mortality among lymph node-positive African-American patients. These deficits would translate to an absolute deficit in 5-year mortality, a commonly referenced landmark measurement in cancer survival, of 1.0%–2.0% for good-prognosis patients (e.g., those who are ER positive and lymph node negative), and of 4.0%–5.0% for patients with a less favorable disease (e.g., ER-negative, lymph node-positive patients). The Survival, Epidemiology, and End Results (SEER)¹ program reports absolute differences in 5-year relative survival (survival corrected for age- and race-specific

life expectancy) of 8% for localized disease and 14% for regional breast cancer (2). Chu et al. (36) studied recent trends in SEER breast cancer mortality among African-Americans and Caucasians, finding that mortality for African-American women leveled off in the middle 1980s for most age groups but has not, as yet, shown the decrease seen among Caucasian women. They speculate that this may be because of a delay in benefit from treatment advances and early detection rather than because of any intrinsic features of breast cancer in African-Americans. The differences in mortality between African-Americans and Caucasians seen among NSABP clinical trial participants who experienced comparable treatment are smaller than those reported in this population-based sample but are, nonetheless, important and warrants further investigation.

Previous results from patients participating in randomized clinical trials have also found generally similar outcomes among different race groups. The Cancer and Leukemia Group B compared characteristics and outcomes for African-Americans and non-African-Americans (Caucasians and others) participating in a trial of adjuvant chemotherapy for lymph node-positive breast cancer (22). The authors found African-Americans to be younger at diagnosis and to have larger tumors that were more often ER negative. Excess risk of death among African-Americans relative to non-African-Americans was reduced from 35% to 14% after taking into account these prognostic factor differences. Excess risk of DFS events for African-Americans was reduced from 24% to 7%. Analyses of patients participating in randomized trials of the Southwest Oncology Group and the Eastern Cooperative Oncology Group also found comparable outcomes between African-Americans and Caucasians (23,24).

Treatment benefits in the trials that we examined here appeared to be commensurate in African-American patients to those of Caucasians. Evidence of differential treatment effects (e.g., interaction between race and treatment group) was not apparent, although such analyses are hindered by low statistical power and, in any case, should be interpreted conservatively (37,38). Both African-American and Caucasian lymph node-negative patients benefited from the addition of chemotherapy or tamoxifen to surgery. Major trial findings among lymph node-positive patients were also similar between African-Americans and Caucasians. These findings are further supported by a recent study (39) of contralateral breast cancer incidence among patients (1212 African-American and 12932 Caucasian) participating in any of nine NSABP trials evaluating treatment for ductal carcinoma *in situ*, stage I, or stage II breast cancer. In that study, patients who received tamoxifen, either as the primary

test question in a trial or incidental to other trial requirements (e.g., some trials mandated tamoxifen for all patients ≥ 50 years of age), were compared with patients who did not receive tamoxifen with respect to contralateral breast cancer incidence. Both African-American and Caucasian patients receiving tamoxifen experienced an approximate 40% reduction in contralateral breast cancer relative to those who did not receive tamoxifen, a result comparable to that observed in NSABP B-14 (30) and the Breast Cancer Prevention Trial (40). While it has been clearly demonstrated that African-American women are more frequently younger at diagnosis and have less favorable tumor characteristics and thus, based on these characteristics, may more frequently qualify as candidates for systemic chemotherapy, it appears that their response to treatment is similar to that of the majority population on which these treatments have been largely developed. The prospect of sufficient racial homogeneity, at least among African-Americans, to result in profound differences in treatment response strictly on the basis of race once known prognostic disease features are accounted for seems to be diminishing, based on results of clinical and genetic epidemiologic studies (41–43).

Observational retrospective studies evaluating outcomes in equal-access health-care systems also suggest equal outcomes among African-American and Caucasian patient populations (21,25,26) or identify additional factors, such as socioeconomic status, that explain remaining outcome differences (44). Results of these studies indirectly provide evidence that, for patients treated in accordance with recommendations for their clinical and pathologic disease presentation, outcomes and extent of benefit among African-Americans and Caucasians are comparable, as seen in clinical trials. Studies of treatment patterns in these settings can also serve to evaluate the extent to which current treatment guidelines are observed in certain patient populations. In some cases, treatment was in accordance with recommended guidelines (45); in contrast, in others, treatment for older and African-American patients did not as frequently adhere to recognized standards (10,14).

The modest mortality deficit noted in these trials is consistent with that seen in other equal-care settings, where breast cancer-specific outcomes were comparable but mortality lagged for African-American women (46,47). In our studies, we speculate that differential background mortality may play a role, particularly in stage I disease, where breast cancer-specific survival prognosis is high. Data from the National Center for Health Statistics (48) indicate a deficit in life expectancy of about 6 years for African-American women, relative to Caucasian women. Even when early mortality is removed, for instance, by examining years of life left for women surviving to 50 years, there remains a shortage of nearly 3 years for African-American women. To what extent these influences might be reflected in comparisons of long-term mortality in clinical trials is unclear. Other studies (49,50) among cancer patients have suggested a significant influence of coexisting diseases on outcomes in terms of both death from competing causes and interference with effective cancer treatment. In the NSABP trials examined for this study, there was a consistent small excess of deaths in the absence of documented recurrence or second primary cancer among African-Americans, suggesting greater mortality from other causes. This was particularly apparent in the trials both among lymph node-negative patients, where breast cancer prognosis is more favorable and, thus, mortality from other causes is

more prevalent, and among lymph node-positive patients with ER-positive tumors, who, in these studies, tended to be older and, thus, prone to greater mortality from other causes in addition to breast cancer. When these “death, no evidence of breast cancer” events were considered censored observations in survival comparisons, the excess risk of mortality for African-Americans was reduced to roughly 8%. Detailed, reliable cause of death information would need to be obtained to fully address this question, since studies (51,52) have indicated problems with using reported cause of death information from clinical trials without careful review. After this information has been obtained, the contribution of non-cancer deaths to observed mortality differences in these trials could be evaluated further. A second possible source of survival differences despite equal time to recurrence is shorter time to death following recurrence among African-Americans, as has been suggested in one study (46). This explanation merits further investigation and will require that that site of recurrence and other factors such as therapy following recurrence be considered. However, it seems unlikely that second-line therapy differences would be a major source of mortality disparities in the randomized clinical trial setting for the same reasons stated earlier.

In summary, African-American and Caucasian women diagnosed at comparable disease stage and appropriately treated tend to experience similar breast cancer prognosis. From the clinical trial data and studies from equal-care settings, it may be indirectly inferred that treatment benefits are comparable across race groups. However, important clinical and pathologic disease characteristics may place certain women at increased risk of poor outcome and warrant continued study of how and why these characteristics may be related to race. Clearly, more comprehensive studies of the growing U.S. populations of women of Asian and Hispanic heritage, as well as the increasingly urban American Indian population, are needed. While the demographic constitution of National Cancer Institute-sponsored clinical trials has been found to be generally representative of the incident cancer burden in the population for the major race classes studied (53), increased racial/ethnic diversity in clinical trial participation is desirable. More diverse participation will provide justification for extrapolating from trial results to the population as a whole, ensure dissemination of quality care in accordance with current treatment guidelines, and provide the necessary data for future investigations of the role of race in breast cancer prognosis and optimal treatment.

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NOTES

¹*Editor's note:* SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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Adjuvant Therapy for Very Young Women With Breast Cancer: Need for Tailored Treatments

Aron Goldhirsch, Richard D. Gelber, Greg Yotlurs, Robert J. Gray, Stephanie Green, John Bryant, Shari Gelber, Monica Castiglione-Gertsch, Alan S. Coates

Breast cancer rarely occurs in women below the age of 35 years. Data from various sources indicate that diagnosis at such an age is associated with a dire prognosis mainly because of a more aggressive presentation. Although the effect of chemotherapy for premenopausal patients is substantial, recent evidence on 2233 patients suggested that very young women with endocrine-responsive tumors had a statistically significantly higher risk of relapse than older premenopausal patients with such tumors. In contrast, results for younger and older premenopausal patients were similar if their tumors were classified as endocrine nonresponsive. Information from studies on 7631 patients who were treated with chemotherapy alone in trials of three major U.S. cooperative groups showed a similar interaction between the effect of age and steroid hormone receptor status of the primary tumor. Better treatments for very young patients are required and may involve ovarian function suppression in addition to other endocrine agents in patients with endocrine responsive tumors and a more precise investigation of chemotherapy and its timing, duration, and intensity in those with endocrine nonresponsive tumors. Very young women with this disease are faced with personal, family, professional, and quality-of-life issues, which further complicate the phase of treatment decision making. The development of more effective therapies for younger patients requires tailored treatment investigations and cannot rely on information predominantly contributed from older premenopausal women. [J Natl Cancer Inst Monogr 2001;30:44-51]

INCIDENCE AND PROGNOSIS

Breast cancer rarely occurs in very young women. About 2% of the patients with the disease are less than 35 years old at diagnosis (1). Below the age of 20 years, the incidence is estimated to be 0.1 per 100 000 women, increasing to 1.4 for women 20-24 years old, 8.1 for women 25-29 years old, and 24.8 for women 30-34 years old (1). Breast cancer at a young age has a more aggressive biological behavior and is associated with a more unfavorable prognosis compared with the disease arising in older premenopausal patients. Specifically, tumors in younger women present with a higher grade and have a higher proliferating fraction and more vascular invasion than those occurring in older patients (2-5). Information from older series indicated that more positive axillary lymph nodes are detected in younger, compared with older, patients. Recent observations at the European Institute of Oncology, Milan, Italy, showed that the proportion of patients with lymph node-positive disease among 185 patients below 35 years of age was similar to that for 1242 patients 35-50 years old treated at the institute between April 1997 and August 2000. Changes in the attention paid to axillary lymph node involvement related to sentinel lymph node work-up

might explain this finding. Results from the same, as yet unpublished, study also indicated that patients under 35 years of age had a higher grade and higher expression of Ki67, a higher percentage of vessel invasion, and less expression of estrogen receptor (ER) and progesterone receptor but similar overexpression of HER2/neu in the primary tumor.

Results from two population-based studies indicate that the risk of death is highest among the youngest and the oldest cohorts when compared with the patients of intermediate age (3), even when the analysis allows for differences in initial tumor stage (5). A review of the National Cancer Data Base (6) reveals that patients younger than 35 years of age have more advanced disease at diagnosis and a poorer 5-year survival than older premenopausal patients. Similar findings have been reported from the National Cancer Institute SEER¹ database (7), from the Finnish Cancer Registry (8), from the Southwest Oncology Group (SWOG) database (9), and from a recent Danish study on young patients who did not receive adjuvant therapy (10), as well as from several series described from single centers (11-13).

Why Focus on Breast Cancer in Women Less Than 35 Years Old?

In addition to considerations related to presentation of disease and prognosis, women under 35 years of age with breast cancer face some specific problems that are less relevant for older premenopausal patients. It is clear, however, that trials reporting results for premenopausal women largely reflect outcomes for patients in their 40s. Table 1 indicates some of the issues that are specific for younger women. These issues include considerations of very late effects of radiation therapy; pregnancy after breast cancer; and interpersonal, family, and professional relations.

Affiliations of authors: A. Goldhirsch, International Breast Cancer Study Group (IBCSG), Bern, Switzerland, European Institute of Oncology, Milan, Italy, and Oncology Institute of Southern Switzerland, Lugano, Switzerland; R. D. Gelber, IBCSG Statistical Center, and Dana-Farber Cancer Institute, Boston, MA; G. Yotlurs, National Surgical Adjuvant Breast and Bowel Project (NSABP) Biostatistical Center and Department of Statistics, University of Pittsburgh, Pittsburgh, PA; R. J. Gray, Eastern Cooperative Oncology Group Statistical Center, Boston, MA, and Dana-Farber Cancer Institute, Boston, MA; S. Green, Southwest Oncology Group Statistical Center and Fred Hutchinson Cancer Research Center, Seattle, WA; J. Bryant, NSABP Biostatistical Center and Departments of Statistics and Biostatistics, University of Pittsburgh, PA; S. Gelber, IBCSG Statistical Center, Boston, and Frontier Science and Technology Research Foundation, Brookline, MA; M. Castiglione-Gertsch, IBCSG Coordinating Center and University of Bern, Switzerland; A. S. Coates, University of Sydney and Australian Cancer Society, Sydney, Australia

Correspondence to: Aron Goldhirsch, M.D., International Breast Cancer Study Group, Department of Medicine, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy (e-mail: agoldhirsch@sakk.ch).

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Table 1. Treatment and personal issues: evidence and current options of approach

Considerations on differences between younger and older premenopausal patients	Status of evidence	Current options (sometimes despite evidence)
Local disease control and very late effects of radiation therapy	Younger patients have a higher risk for locoregional relapse (45,46). No data are available on late effects of anthracyclines and taxanes plus radiation therapy on the heart.	Breast conservation with radiation therapy is considered a standard (47). Total or bilateral (prophylactic) mastectomy is increasingly discussed (48).
Pregnancy after breast cancer	Pregnancy seems to be safe after breast cancer and after adjuvant systemic cytotoxic therapy (49,50,51) (except for BRCA1 and BRCA2 carriers) (52). Uncertainty exists concerning pretreatment with tamoxifen and neonatal genital tract malformations (53).	There is reluctance to consider pregnancy even for women with lymph node-negative disease (54). Gonadotropin-releasing hormone analogue is available as an effective endocrine treatment, especially if given with tamoxifen (55). (New endocrine therapies are being investigated, mainly in postmenopausal patients.)
Interpersonal and family relations; professional decisions	Younger women might be particularly vulnerable to the emotional distress of the disease (56) because they need to face disease and treatments before attaining many personal accomplishments.	Psychological support is an option that is being tested in trials.

Age and Chemotherapy-Induced Amenorrhea

Current adjuvant chemotherapy, which is extensively used across the board in premenopausal patients because of its overwhelming effects on outcome (14), is less likely to have definitive endocrine effect (suppression of endocrine ovarian function) in women younger than 35 years old. This is a major issue influencing the selection of adjuvant therapies for very young patients. Table 2 displays the incidence of chemotherapy-induced amenorrhea in 1054 patients treated with a combination of cyclophosphamide, methotrexate, and 5-fluorouracil (classical CMF) for three to nine courses. No endocrine therapy was prescribed. All patients had lymph node-positive breast cancer (15).

In this cohort, some of the patients who had amenorrhea subsequently resumed menses, so that only 8% of the younger patients, compared with 59% of the older patients, had permanent amenorrhea. Furthermore, the incidence of ovarian endocrine suppression is proportional to the duration of chemotherapy (16). It is known that chemotherapy exerts some of its effects in this age group via endocrine mechanisms (17).

Acceptance of ovarian function suppression is a significant problem for the younger patients (18,19). Facing objective and subjective symptoms of menopause, psychological distress, and the potential need to adjust to changes in personal and family plans requires specific attention. Chemotherapy also seems easier to offer (in terms of acceptance) to the younger patients because of its shorter duration and the lesser degree of long-term effects on endocrine functions. These aspects require specific investigations focusing on the youngest cohorts.

EFFECTS OF AVAILABLE SYSTEMIC TREATMENTS

Typically, young patients receive chemotherapy, and in many countries, clinicians have been reluctant to employ ovarian ab-

lation or other endocrine treatment (10). The Danish Group studied patients enrolled in clinical trials between 1977 and 1996 with respect to age. During this period, they observed 867 patients (8.4%) who were less than 35 years old and 9489 patients who were 35–49 years old. Their analysis was based on the recommendations from the St. Gallen Conference (20), which indicated that an age less than 35 years is a dire prognostic variable. Patients with a lower risk of relapse (based on lymph node status, tumor size, and histologic grade but not hormone receptor status) were untreated with adjuvant systemic therapy, while the high-risk patients were offered participation in trials with chemotherapy or endocrine therapy or a combination of both (10). In this study, among patients who were predefined as having a low-risk disease and who, therefore, were given no adjuvant systemic treatment, the youngest cohort had a significantly increased risk of dying compared with older women. The increased risk with decreasing age at diagnosis (adjusted relative risk [RR] with the 45- to 49-year-old cohort as the reference group having an RR of 1.00) was 1.12 (95% confidence interval [CI] = 0.89 to 1.40) for the 40- to 44-year-old group, 1.40 (95% CI = 1.10 to 1.78) for the 35- to 39-year-old group, and 2.18 (95% CI = 1.64 to 2.89) for the under 35-year-old group. No such trend according to age was seen in patients who were considered to have high-risk disease and who were, therefore, eligible to receive adjuvant cytotoxic treatment. Thus, the negative prognostic effect of young age (<35 years old) compared with patients who were 45–49 years old was confined to those who were not offered a trial including adjuvant cytotoxic treatment. This led to the conclusion that young women with breast cancer, on the basis of age alone, should be regarded as high-risk patients and should be given adjuvant cytotoxic treatment. This latter conclusion relies on the assumption that the worse prognosis predicts responsiveness to chemotherapy. Information on differences in treatment effects according to steroid hormone receptor status of the primary tumor was not reported in this analysis.

The International Breast Cancer Study Group (IBCSG) (formerly the Ludwig Breast Cancer Study Group) reported previously on 3700 premenopausal and perimenopausal patients who were included in IBCSG Trials I (21), II (22), V (23,24), and VI (15) conducted by the group between 1978 and 1993 (25). Of these women, 314 (8.5%) were less than 35 years old at study entry. Relapse and death occurred earlier and more often in

Table 2. Endocrine effects of chemotherapy in premenopausal patients—percentage of patients with amenorrhea for at least 3 months in International Breast Cancer Study Group Trial VI according to age (15)

Age group, y	No. of patients (%)	No amenorrhea, %	Amenorrhea followed by resumption of menses, %	Permanent amenorrhea, %
<35	90 (8.5)	88	4	8
≥35	964 (91.5)	34	7	59

younger (<35 years old) than in older (≥ 35 years old) patients. The 10-year disease-free survival (DFS) for younger patients was 35% versus 47% for older patients ($P < .001$), and the 10-year overall survival (OS) was 49% versus 62% ($P < .001$), respectively. Younger patients with ER-positive tumors had a significantly worse prognosis than did younger patients with ER-negative tumors (10-year DFS was 25% for ER-positive tumors versus 47% for ER-negative tumors; $P = .014$). In contrast, among older patients, the prognosis was similar for patients with ER-positive tumors compared to patients with ER-negative tumors (10-year DFS was 45% versus 46%; $P = .27$). The interaction between age and ER status on outcome was statistically significant ($P = .002$). Of the 3098 patients with known ER status, 2233 (72%) received at least three courses of adjuvant chemotherapy alone. This retrospective analysis suggests that the endocrine effects of chemotherapy alone were insufficient for the younger patients with endocrine-responsive breast cancer.

Adjuvant Chemotherapy Alone and Outcome According to Age

To investigate further the hypothesis that there exists an interaction between age and ER status in premenopausal women treated with chemotherapy alone, the major U.S. cooperative groups were invited to conduct a similar analysis in their trial populations. The National Surgical Adjuvant Breast and Bowel Project (NSABP), the Eastern Cooperative Oncology Group (ECOG), and the SWOG provided information on outcome of patients assigned to receive chemotherapy alone within premenopausal age cohorts—premenopausal patients for ECOG and SWOG and patients aged 49 years and younger for NSABP. In addition, the IBCSG analysis was restricted to patients assigned to receive at least three courses of classical CMF, a duration similar to four courses of a doxorubicin (Adriamycin)–cyclophosphamide (AC) regimen, an adjuvant chemotherapy program frequently used in U.S. trials. All patients had a known ER status. From IBCSG trials, 2233 patients met these criteria

(15,21–23). Included were 5849 patients 49 years of age or younger whose ER status was ascertained and who were randomly assigned to receive chemotherapy alone in NSABP trials that included both ER-positive and ER-negative cases [B-06 (26,27), B-09 (28), B-11 (29), B-15 (30), B-18 (31), B-22 (32), and B-25 (33)]. Also included were 1112 premenopausal patients assigned to receive chemotherapy alone in the ECOG trials EST5177 (34), EST5188 (35), and EST3189 (36) and 670 premenopausal patients assigned to receive chemotherapy alone in SWOG trial S8897 (37) for lymph node-negative disease.

Table 3 summarizes the results from all four cooperative groups. In each case, the RR of an event, estimated from a Cox proportional hazards regression model stratified by study and treatment group, is substantially higher for young patients with ER-positive tumors compared with the reference population of older patients with ER-positive tumors. In contrast, the difference in outcome with respect to age group is much smaller for patients with ER-negative tumors. The interaction between age and ER status was statistically significant for the cohorts in the IBCSG, NSABP, and SWOG trials. The bottom portion of Table 3 displays the 5-year DFS percentages calculated by applying the RR to the Kaplan–Meier estimates obtained for the reference population.

Fig. 1 shows the Kaplan–Meier plots of DFS (IBCSG, SWOG) and relapse-free interval (NSABP) for ER-positive versus ER-negative cohorts separately for younger (<35 years old) and older (≥ 35 years old) premenopausal women enrolled in chemotherapy-only treatment groups. Curves are not entirely appropriate for the ECOG dataset because of the confounding of treatment, lymph node status, and ER status in the different patient cohorts. The plots from the other three groups reveal a consistent pattern confirming a worse outcome for young patients with ER-positive tumors who are treated with chemotherapy alone. The nonproportionality of hazards for ER-positive and ER-negative cohorts is also evident, indicating that the RR in Table 3 should be interpreted as average hazards over the time interval.

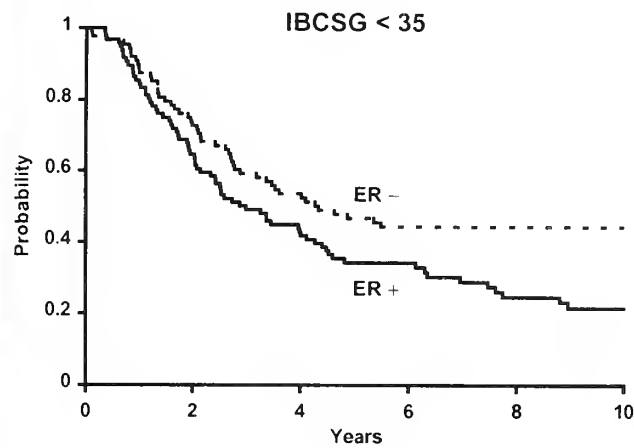
Table 3. Relative risk of relapse* and corresponding 5-year disease-free survival* for premenopausal women in chemotherapy-only groups in trials conducted by IBCSG, NSABP, ECOG, and SWOG†

Group	Total patients	ER positive		ER negative		Interaction <i>P</i>
		<35 y	≥35 y‡	<35 y	≥35 y‡	
<i>Relative risk of relapse (No. of events/No. of patients)</i>						
IBCSG	2233	1.84 (72/96)	1.00 (referent) (737/1353)	1.13 (50/88)	1.02 (370/696)	.009
NSABP	5849	1.72 (254/402)	1.00 (referent) (1210/2716)	1.27 (214/441)	1.12 (1045/2290)	.0001
ECOG	1112	1.54 (42/71)	1.00 (referent) (274/602)	1.40 (40/73)	1.26 (195/366)	.17
SWOG	670	2.67 (11/29)	1.00 (referent) (48/293)	0.81 (7/55)	1.13 (52/293)	.012
<i>5-y disease-free survival, %*</i>						
IBCSG		39	60	56	59	
NSABP		49	66	59	63	
ECOG		53	66	55	58	
SWOG		73	89	91	88	

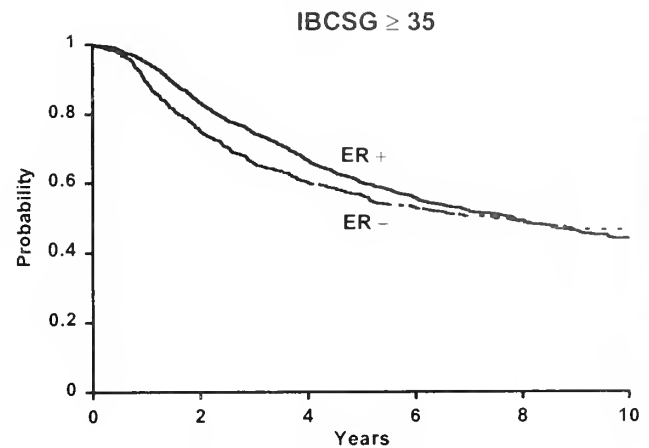
*Includes breast cancer relapses, second primary breast tumors, and deaths without relapse for IBCSG (also includes non-breast second primaries), ECOG, and SWOG; includes only breast cancer relapses (other events are censored) for NSABP.

†Cohorts defined by age and estrogen-receptor (ER) status are compared with the reference population of older women with ER-positive tumors (number of events/number of patients are shown in parentheses). IBCSG = International Breast Cancer Study Group; NSABP = National Surgical Adjuvant Breast and Bowel Project; ECOG = Eastern Cooperative Oncology Group; and SWOG = Southwest Oncology Group.

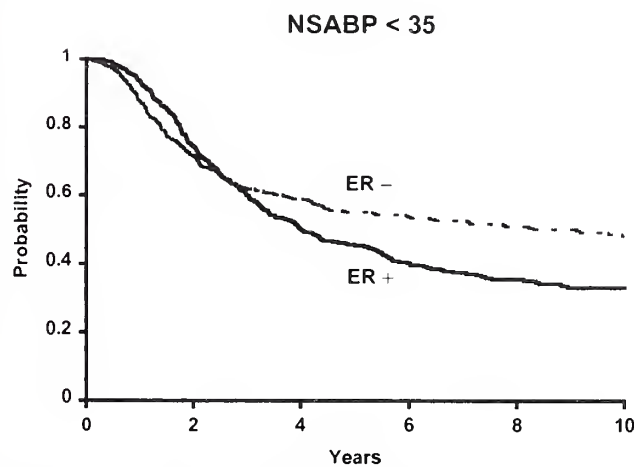
‡Premenopausal women ≥ 35 years old for IBCSG, ECOG, and SWOG; 35–49 years old for NSABP.



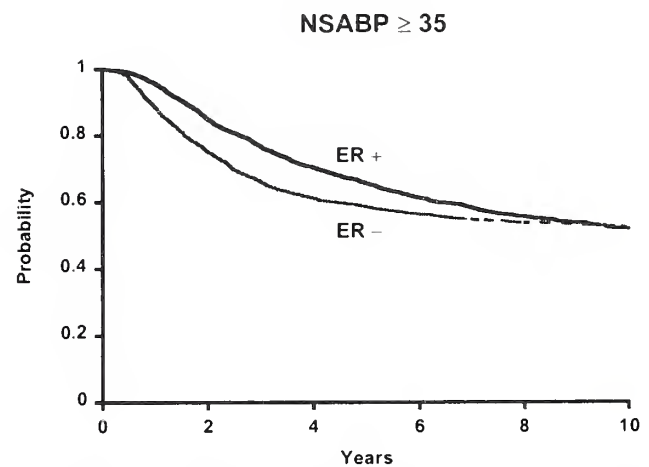
ER +	96	62	41	28	17	12
ER -	88	64	47	38	28	20



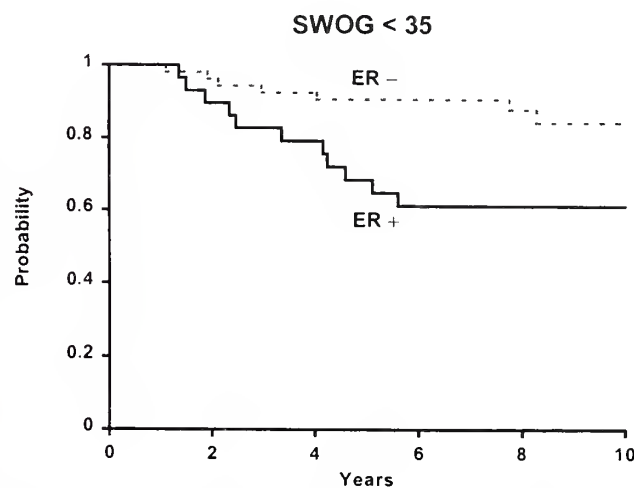
ER +	1353	1126	897	654	389	214
ER -	696	521	415	311	225	158



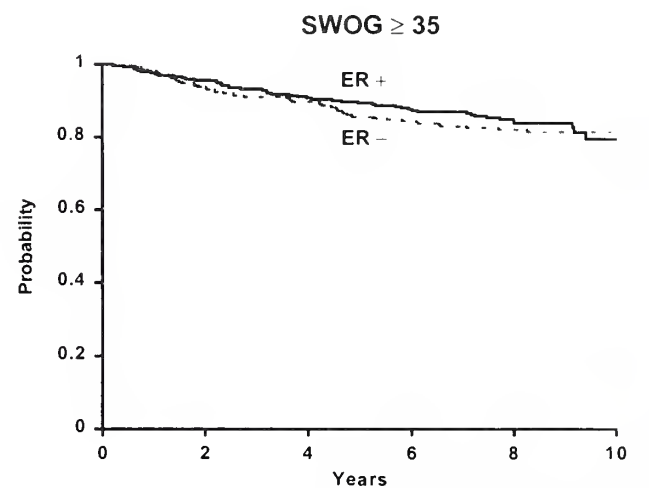
ER +	403	293	192	130	84	50
ER -	442	308	247	176	133	86



ER +	2721	2265	1820	1375	921	544
ER -	2294	1694	1333	1019	730	493



ER +	29	26	22	17	12	0
ER -	55	52	49	47	28	5



ER +	293	280	265	249	165	13
ER -	293	274	260	237	145	15

Fig. 1. Kaplan-Meier plots of disease-free survival (IBCSG, SWOG) and relapse-free survival (NSABP) for estrogen receptor (ER)-positive versus ER-negative cohorts separately for younger (<35 years old) and older (≥ 35 years old) premenopausal women enrolled in chemotherapy-only treatment groups.

Is failure to achieve chemotherapy-induced amenorrhea associated with an increased risk of relapse among premenopausal patients with ER-positive tumors? Table 4 shows the RR of relapse comparing patients with no amenorrhea versus those with amenorrhea treated with at least three cycles of classical CMF on IBCSG trials (25). A landmark analysis was used excluding patients who had relapsed or died within 9 months of randomization. Amenorrhea was defined as cessation of menses at each of the 3-, 6-, and 9-month follow-up visits for Trials I, II, and V or at the 9-month follow-up visit for women in Trial VI. Subgroups of patients defined by age and ER status are shown in Table 4. For both young and older premenopausal women with ER-positive tumors, no amenorrhea is associated with a higher risk of relapse, although the result in younger women is statistically uncertain because of the small sample size. The difference in the proportion of patients who achieve amenorrhea in the young age group might contribute to the poor outcome of these women with endocrine-responsive tumors treated with chemotherapy alone. In contrast, the association between failure to achieve chemotherapy-induced amenorrhea and risk of relapse is not statistically significant for patients with ER-negative primaries. Whether treatments that provide ovarian function suppression should be offered to women who continue to menstruate following adjuvant chemotherapy requires study in a randomized clinical trial.

Various Adjuvant Approaches and Outcome According to Age

What evidence exists concerning differences in outcome according to age for premenopausal patients who receive other types of adjuvant therapies? Table 5 shows the RR of relapse between age groups less than 35 years and those 35 years old and older on the basis of data from the four cooperative groups. An RR greater than 1.00 indicates that the younger patients have a higher risk of relapse when compared with the older patient cohort. In addition to the NSABP trials that included the chemotherapy-only treatment groups presented in Table 3, Table 5 displays data from treatment groups allocated to no adjuvant therapy [from B-06 (26,27), B-13 (38), and B-14 (39)], treatment groups allocated to tamoxifen alone given for 5 years [from B-14 (39), B-16 (31), and B-20 (40)], and treatment groups allocated to chemotherapy plus tamoxifen [from B-09 (28), B-12 (29), B-20 (40), and B-23 (41)]. The ECOG data for the chemotherapy plus tamoxifen treatment group come from the cyclophosphamide, methotrexate, 5-fluorouracil, prednisone, and tamoxifen (CMFPT) arm of EST5177 (34) and from all patients enrolled in EST5181 (42). The SWOG data for the chemo-

therapy plus tamoxifen treatment group come from the same S8897 study (37) that provided the data for the chemotherapy-alone group. ER-positive disease is shown in the upper part of Table 5, and ER-negative disease is shown in the lower part.

The interaction with ER status described for the chemotherapy group is evident—RR of relapse for younger women compared with older women is higher for ER-positive cohorts compared with ER-negative cohorts. Differences in outcome according to age for no treatment and for chemotherapy plus tamoxifen are very similar, and interactions with ER status are not statistically significant. The large RR for tamoxifen given alone for 5 years for the ER-positive cohort is noteworthy: Younger premenopausal patients do worse than older premenopausal women when treated with tamoxifen alone. Whether tamoxifen alone improves outcome compared with no adjuvant treatment for both younger and older premenopausal women requires evaluation within the randomized studies. A priority research question is whether endocrine ovarian suppression, added to tamoxifen with or without chemotherapy, might improve outcome. Such a treatment approach has been demonstrated to be effective in a single trial in advanced breast cancer (43).

For the ER-negative cohort displayed in Table 5, the beneficial effects of chemotherapy might be similar for younger and older premenopausal women. In fact, in Trial B-13 (38), which is specifically designed for ER-negative disease, the effect of chemotherapy compared with no adjuvant treatment in women less than 50 years old is overwhelming, corresponding to a 38% reduction in the risk of relapse (Table 6). In this setting of ER-negative disease, the magnitude of the estimated effect of chemotherapy is the same for younger as for older patients although, because of the smaller sample size, the result for the younger group is statistically uncertain.

Endocrine Nonresponsive Tumors

Regardless of the age of premenopausal patients with ER-negative tumors, adjuvant chemotherapy appears to be a very important component of a successful treatment regimen. It is hypothesized that questions relating to the direct cytotoxic effects of chemotherapy should be investigated specifically in patients with endocrine-nonresponsive tumors without the confounding effects of hormonal therapies or endocrine effects of chemotherapy. For example, timing of the start of chemotherapy after surgery; type, schedule, and duration of the cytotoxic regimen; and especially dose escalation and dose density of chemotherapy should be investigated in this patient population. ER-negative status is the typical criterion for defining endocrine nonresponsive tumors in clinical trials and in the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) overview. A large number of markers (e.g., HER2/neu, p53, etc.) might

Table 4. 9-month landmark analysis of premenopausal patients with lymph node-positive disease treated with at least 3 months of classical CMF* on International Breast Cancer Study Group Trials: relative risk of relapse comparing patients with no amenorrhea at 9 months versus patients with chemotherapy-induced amenorrhea at 9 months

Age, y, and receptor status	No. of patients		Relative risk	95% confidence interval	P
	No amenorrhea	Amenorrhea			
<35, estrogen receptor (ER) positive	61	21	1.53	0.80 to 2.94	.20
≥35, ER positive	320	773	1.34	1.12 to 1.61	.0014
<35, ER negative	54	23	1.14	0.42 to 3.15	.79
≥35, ER negative	152	387	1.11	0.85 to 1.45	.45

*CMF = combination of cyclophosphamide, methotrexate, and 5-fluorouracil.

Table 5. Relative risk of relapse comparing patients less than 35 years old with those 35 years of age and older

	No. of patients	No. of events	Relative risk (<35 versus ≥35 y old)	95% confidence interval	P
Estrogen receptor (ER) positive					
Chemotherapy					
IBCSG	1449	809	1.88	1.47 to 2.39	.0001
NSABP	3118	1464	1.79	1.56 to 2.05	.0001
ECOG	673	316	1.60	1.16 to 2.22	.005
SWOG	322	59	2.72	1.41 to 5.24	.003
No treatment					
NSABP	643	206	1.59	1.07 to 2.36	.02
Tamoxifen					
NSABP	792	178	1.91	1.21 to 3.01	.006
Chemotherapy + tamoxifen					
NSABP	1197	330	1.55	1.14 to 2.12	.006
ECOG	499	259	1.94	1.40 to 2.69	.0001
ER-negative					
Chemotherapy					
IBCSG	784	420	1.10	0.82 to 1.48	.54
NSABP	2731	1259	1.11	0.96 to 1.29	.16
ECOG	439	235	1.10	0.78 to 1.54	.59
SWOG	348	59	0.71	0.32 to 1.56	.39
No treatment					
NSABP	390	137	1.31	0.85 to 2.01	.22
Tamoxifen					
No data	—	—	—	—	—
Chemotherapy + tamoxifen					
NSABP	707	178	1.33	0.92 to 1.91	.13
ECOG	367	224	1.39	1.01 to 1.90	.04
SWOG	365	76	0.96	0.53 to 1.74	.89

*IBCSG = International Breast Cancer Study Group; NSABP = National Surgical Adjuvant Breast and Bowel Project; ECOG = Eastern Cooperative Oncology Group; SWOG = Southwest Oncology Group.

Table 6. Relative risk of relapse comparing patients in the chemotherapy group (M → F) versus no adjuvant therapy (nil)—results from National Surgical Adjuvant Breast and Bowel Project Trial B-13 for estrogen receptor-negative, lymph node-negative cases

Age group, y	No. of patients	No. of events	Relative risk M → F versus nil	95% confidence interval	P
<35	69	28	0.62	0.29 to 1.30	.21
35–49	371	107	0.62	0.42 to 0.91	.01

prove to be useful in the future to refine the selection of chemotherapy for patients whose tumors are exclusively affected by cytotoxic agents (44).

Endocrine Responsive Tumors

We observed from several trials conducted during the past two decades that younger premenopausal patients treated with chemotherapy alone have a higher risk of relapse and death than older premenopausal women treated in the same way, especially if their tumors express hormone receptors. For young patients whose tumors express hormone receptors, endocrine effects of chemotherapy alone are modest, and endocrine therapies appear to be an essential component of an effective adjuvant therapy program. Whether use of “optimal” endocrine therapy (e.g., ovarian function suppression plus tamoxifen) may be sufficient for these patients is a hypothesis that has not been tested adequately. It must be recognized that factors influencing acceptance of endocrine therapies by very young women are complex, involving issues such as treatment duration (typically several years), induced menopausal symptoms, and issues of sexual functioning and family planning.

CONCLUSIONS

A significant proportion of the youngest patients with breast cancer have a dire prognosis, in part, because of a more aggressive presentation of the disease. The belief that an increased risk of relapse justifies use of cytotoxic agents to increase cancer cell kill and the demonstration of significant chemotherapy efficacy for premenopausal women (most of whom are >34 years of age) contributed to lack of progress in evaluating endocrine therapies for this rare presentation of breast cancer. Furthermore, treatment decision making for very young women with newly diagnosed breast cancer may be affected by the strong emotional involvement of care providers.

There is an urgent need for tailored treatment investigations, especially in this younger population, for whom chemotherapy is prescribed across the board. Endocrine therapies, which are not easy to offer to very young patients, must be investigated in hormone-responsive disease because substantial evidence exists that current approaches are suboptimal. We must improve our understanding about how best to use endocrine approaches, including ovarian function suppression, use of selective estrogen receptor modulators (SERMs) and other endocrine agents, and possibly timing of surgery with respect to the phase in the menstrual cycle.

We might also consider reinvestigating questions related to chemotherapy in younger patients: Questions of timing, duration, and intensity of chemotherapy might be answered more precisely in patients with endocrine nonresponsive tumors, for whom endocrine effects of chemotherapy do not confound our observations.

Questions of endocrine therapies (tamoxifen or ovarian suppression or the combination of both) and of cytotoxic agents should also be considered with respect to the patient's desire to

become pregnant and with respect to the presence of BRCA1 and BRCA2 mutations. These relatively rare conditions were not studied prospectively in the past, and a focused investigation might result in treatment indications, which cannot otherwise be extrapolated from trials on an older population.

Accrual of younger women to past and current clinical trials is insufficient to allow significant progress on treatment of these patients. Prospectively designed global collaboration to specifically investigate therapeutic approaches for young patients with breast cancer is required.

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NOTES

¹*Editor's note:* SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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Factors Used to Select Adjuvant Therapy of Breast Cancer in the United States: an Overview of Age, Race, and Socioeconomic Status

Hyman B. Muss

Age, race, and socioeconomic status all play a role in decisions regarding breast cancer adjuvant therapy. Increasing age remains the major risk factor for breast cancer, while in very young women breast cancer may have a poorer prognosis, even when adjusted for disease stage and other variables. More than half of all new breast cancers in the United States occur in women older than 65 years. Because of the higher frequency of coexisting (comorbid) serious illness in older women, the benefits of adjuvant therapy get smaller as age increases. Adjuvant therapy with tamoxifen and/or chemotherapy can statistically significantly improve survival in older women, but older women are less likely to receive chemotherapy and are less likely to be offered participation in clinical trials. Efforts are now under way to overcome age bias among health care providers and to develop clinical trials focusing on older patients. Breast cancer mortality is higher in African-Americans than in white Americans. Although the biologic characteristics of breast cancer are worse in African-Americans, major differences in survival are related to socioeconomic factors and access to care. When matched for disease stage and other major clinical and biologic variables, African-American and white patients have similar survival rates. Few data are available on the effects of adjuvant treatment on early breast cancer outcomes in Hispanic Americans and Asian-Americans. Poverty and lack of insurance are surrogates for poor outcomes; major efforts are needed to guarantee all Americans high-quality cancer care. [Monogr Natl Cancer Inst 2001;30: 52-5]

Age, race, and socioeconomic status all affect decisions regarding breast cancer adjuvant therapy. More than half of all new breast cancers in the United States occur in women 65 years old or older, a statistic that has even more impact in a population whose longevity is increasing (1). Since coexisting illness (comorbidity) statistically significantly increases with age, it is a major factor in determining the relative benefit of adjuvant therapy on patient survival (2). In younger women, especially those less than 35 years old, breast cancer may have a poorer prognosis, even when adjusted for disease stage and other variables (3,4). African-Americans now constitute 12.7% of the U.S. population and have higher breast cancer mortality compared with Caucasian Americans, even after adjustment for disease stage (5). Such differences are related to several factors, including stage at presentation, tumor biology, and sociodemographic characteristics (5). Few data are available concerning breast cancer outcomes in Hispanic Americans who constitute 8.2% of the population, Asian-Americans who constitute 1.6% of the population, and Native Americans who constitute almost 1% of the population. In one study of 163 African-American, 205 His-

panic, and 964 Caucasian women, lower socioeconomic status was the major factor related to the poorer survival rates of Hispanic and African-American women compared with Caucasian women (6).

RACE AND SOCIOECONOMIC STATUS AS A FACTOR IN ADJUVANT THERAPY OF BREAST CANCER

Several trials have shown small but potentially important biologic differences in breast cancer among African-American and Caucasian patients. African-American patients are more likely than Caucasians to have more biologically aggressive tumors, including larger tumor size, an increased likelihood of involved lymph nodes, lack of hormone receptors, and a higher rate of tumor proliferation (Table 1) (7). Hispanic Americans had tumor characteristics somewhat worse than Caucasians but better than African-Americans in this series. These adverse biologic tumor characteristics may limit the potential life-prolonging benefits of tamoxifen therapy or ovarian ablation in African-American and Hispanic patients compared with Caucasians. In large numbers of patients, these small differences in tumor biology may prove to be highly meaningful.

African-Americans and Hispanics do appear to be adequately represented in clinical trials (8). Limited data suggest that, at least for African-Americans, the benefits of adjuvant therapy are similar to those for Caucasian women when outcomes are adjusted for disease stage, comorbid illness, and pathologic and sociodemographic variables (9,10); however, most studies have not had the power to adequately test whether specific treatments have different survival and response rates in minority populations compared with the study group as a whole. Few data are available on Hispanic patients and other minorities concerning the risks and benefits of adjuvant therapy. The data also suggest that for Hispanics, like African-Americans, socioeconomic factors play a key role in outcome (6).

Poverty is associated with poorer cancer outcomes for all Americans, irrespective of racial or ethnic group, and remains a national issue (11). In one sobering study of 4675 women aged 35-64 years with breast cancer entered in the New Jersey State Tumor Registry, the adjusted risk of death was 49% higher for uninsured patients and 40% higher for Medicaid patients than for privately insured patients during the 54-89 months after diagnosis, even after adjustment for age, race, household income, coexisting illness, and disease stage (12). These numbers are of major concern, since 40-50 million Americans are cur-

Correspondence to: Hyman B. Muss, M.D., University of Vermont College of Medicine, Vermont Cancer Center, University Health Center, St. Joseph 3400, 1 South Prospect St., Burlington, VT 05401 (e-mail: hyman.muss@vmednet.org).

See "Note" following "References."

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Table 1. Differences in tumor biology among different racial and ethnic groups*

	Caucasian, %	Hispanic, %	African-American, %
Age, ≤50 y	23.9	39.0	37.4
Tumor size, >2 cm	55.5	67.9	70.3
Lymph node positive	42.1	54.1	51.2
Estrogen receptor positive	56.0	50.6	40.8
High S phase	38.5	48.4	49.4
HER2+	16.1	20.9	13.8

*Thirty-one hospitals in the United States. Modified from Elledge et al. (7).

rently underinsured or uninsured during a time of unprecedented economic growth. Providing access to high-quality health care for poorer and uninsured Americans remains a major national dilemma. Major efforts by the U.S. National Cancer Institute and other organizations to improve access of minorities to clinical trials are under way.

These data suggest that, at least for African-Americans, access to high-quality adjuvant treatment leads to similar outcomes compared with Caucasian patients, provided the patients are given similar treatments and have similar tumor biologic characteristics. However, differences in tumor biologic characteristics among African-Americans and Caucasian indicate that, even if care and access were equal, the unfavorable tumor characteristics more often seen in African-American patients would still result in a higher overall mortality for this group. Further research concerning the underlying molecular events leading to these differences in breast tumor biology is needed.

AGE AS A FACTOR IN ADJUVANT THERAPY OF BREAST CANCER

Breast cancer incidence increases dramatically with age. In the United States, the incidence of breast cancer increases from less than 100 per 100,000 women in women less than 45 years old to a risk exceeding 300 per 100,000 women in women 75 years old or older (Fig. 1) (13). Mortality data follow a similar pattern, with the majority of breast cancer deaths occur-

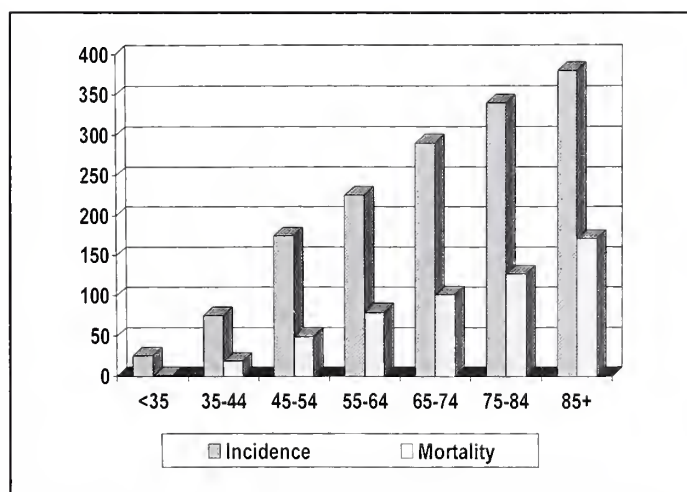


Fig. 1. Incidence of and mortality from breast cancer and age [modified from Yancik et al. (13)]. The x-axis shows incidence (per 100,000); the y-axis shows age range in years.

ring in women 60 years old or older. Similar statistics are found in other industrialized countries. Since the populations of the United States and other industrialized nations continue to age, breast cancer is likely to be a more prevalent and common disease in the future (14) Goldhirsch et al. (4) in this monograph have presented compelling data that, for very young women, breast cancer carries a poorer prognosis even after adjustment for disease stage and other clinically important prognostic factors. Such data suggest that clinical trials in these young patients should explore more aggressive and, hopefully, more beneficial therapies than current standard treatments.

Breast cancer biology changes with age, and older patients have more favorable tumor characteristics. Diab et al. (15) compared the tumor and biologic characteristics of breast cancer patients among age groups (Table 2). These more favorable tumor characteristics may translate into improved survival; for lymph node-negative patients in this series, survival was similar among breast cancer patients 70 years old or older and age-matched women from the general population. Nevertheless, several trials have shown that older women are less likely to receive appropriate local therapy, such as postoperative local radiation, or adjuvant systemic therapy compared with younger women (16). In healthier older women with higher risk lymph node-negative and lymph node-positive breast cancer, however, such undertreatment may lead to poorer outcomes.

Compelling data from a worldwide meta-analysis of adjuvant therapy (17) showed that, for older patients with estrogen receptor-positive or progesterone receptor-positive tumors, tamoxifen statistically significantly increased both relapse-free and overall survival. Women 70 years old or older who took tamoxifen for 5 years had a 54% decrease in the annual odds of breast cancer recurrence and a 34% decrease in the annual odds of dying of breast cancer. Chemotherapy alone has not been studied adequately in older patients; in the same overview, fewer than 700 women 70 years old or older were entered in randomized trials. Chemotherapy, however, was associated with statistically significant improvements in both relapse-free and overall survival in women aged 50–69 years (a 20.3% and 11.3 % reduction in annual odds of relapse and death, respectively) (18). It is reasonable to expect that chemotherapy would have a similar effect in women 70 years old or older, but more data on chemotherapy trials are needed in older women.

The effects of major coexisting illness on survival are a crucial factor in assessing the potential benefits of chemotherapy in older patients. The potential benefits of adjuvant therapy in older women have been estimated recently with the use of a mathematical model (19); it is clear that the value of adjuvant therapy diminishes substantially as age and comorbidity increase and as

Table 2. Differences in tumor biology with increasing age*

	Age, y			
	55–64	65–74	75–84	≥85
No. of patients	12 101	13 123	7873	2018
Lymph node negative, %	59	65	66	61
Estrogen receptor positive, %	83	87	90	91
Low S phase, %	51	57	61	60
HER2+, %	21	15	14	10

*All differences are statistically significant across groups ($P<.001$). Modified from Diab et al. (15).

non-breast cancer-related illness becomes a major competing cause for death. What is also clear is that older women in good general health tolerate standard chemotherapy regimens almost as well as younger women (20,21). Outside a trial, recommendations for adjuvant therapy made by an international consensus panel appear prudent and should be used as a treatment guideline (Table 3) (22).

Comorbidity is frequently a key issue in the decision to use adjuvant systemic therapy in the elderly. As comorbidity increases, the potential benefits of adjuvant therapy decrease, since the risk of dying of other non-breast cancer-related causes becomes more likely. In one study, women with three or more comorbid illnesses had a 20-fold higher risk of dying of non-breast cancer-related causes than women with no comorbid conditions, even after adjustment for disease stage, type of treatment, race, and social and behavioral factors (23). Several investigators (2,19,24) have developed models to predict the likely benefit of age or comorbidity on treatment outcome in older women with early-stage breast cancer. These models clearly show that the benefits of adjuvant therapy decrease with increasing age or comorbidity. Welch et al. (25) also provided data derived from a Markov model that predicted the effect of comorbidity on life expectancy (Fig. 2). Even with potentially curative treatment, the effect of such treatment on overall life expectancy would be small for very old patients.

It is clear that older patients are underrepresented in clinical trials (8,26). Hutchins et al. (8) analyzed data from 16 396 patients consecutively enrolled in 164 Southwest Oncology Group treatment trials during the period from 1993 to 1996. Only 9% of the patients entered in breast cancer clinical trials were 65 years old or older compared with population and census estimates that indicated 49% of women with breast cancer during this time period were 65 years or older (8). Barriers to the participation of older women in clinical trials have been well defined by Trimble et al. (26) and include the following: 1) a research focus on aggressive therapy, which may be unacceptably toxic to the elderly; 2) the presence of comorbidity; 3) fewer trials specifically aimed at older patients; 4) limited expectations for long-term benefits on the part of physicians, relatives, and the patients themselves; and 5) a lack of financial, logistic, and social support for the participation of elderly patients in clinical trials (26). Issues related to reimbursement may be partially overcome by the recent Presidential mandate to the Center for Medicare and Medicaid Services to reimburse the costs of routine care for patients entered in appropriately reviewed clinical trials. Moreover, a recent study from the Cancer and Leukemia Group B (27) compared participation in clinical trials among patients with stage I, II, or IV breast cancer who were less than 65 years with that among patients who were 65 years old or older who were treated by the same physician and who were eligible for a clinical trial within their institution. Of 77 pairs of patients matched

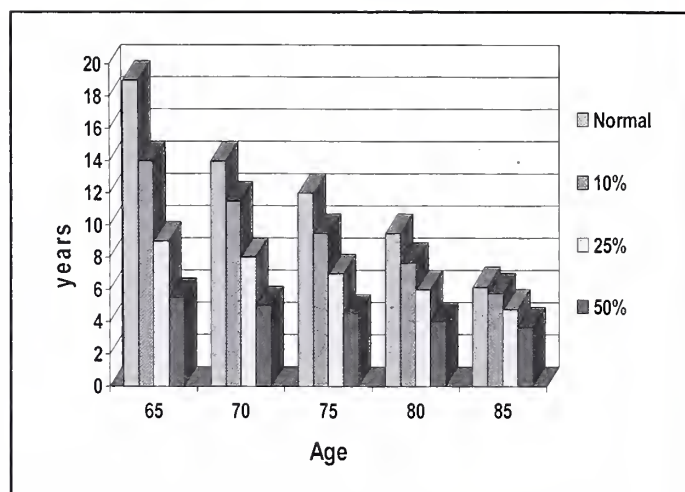


Fig. 2. Effects of coexisting illness (comorbidity) on life expectancy [modified from Welch et al. (25)]. Normal = no major coexisting illness, 10% = illness with 10% mortality rate at 5 years, 25% = illness with 25% mortality rate at 5 years, and 50% = illness with 50% mortality rate at 5 years. The y-axis shows life expectancy in years. For example, for an 85-year-old woman with no illness and no breast cancer diagnosis, life expectancy is 6 years. For an 85-year-old woman diagnosed with stage I breast cancer with an expected 10% mortality rate at 5 years, the life expectancy would be just under 6 years. Even without systemic therapy added, the breast cancer does not substantially alter her life expectancy, and the addition of systemic therapy would have no major benefit.

on the basis of disease stage and treating physician, 34% of those 65 years old or older were offered trial participation compared with 51% younger than 65 years ($P < .05$). When participation in a trial was offered, however, similar rates of acceptance were found in the older and younger women (50% and 56% for older and younger women, respectively). Even after adjustment for other covariates, including race, comorbidity, educational level, marital status, and satisfaction with care, age alone predicted for a lower likelihood of being offered a clinical trial. Efforts are now under way to overcome these barriers and include National Cancer Institute and National Institute of Aging funding for clinical trials directed at older women (Table 4) (28).

CONCLUSIONS

Racial, ethnic, and aging issues play a major role in appropriate delivery of adjuvant therapy in women with early-stage breast cancer. Although there are differences in tumor biology related to age and race, the major issue related to adjuvant therapy is access to care. For African-American and other ethnic and racial groups, socioeconomic factors are far more important than biologic factors in determining outcome. Lower incomes, limited or absent insurance coverage, and poorer education all translate into less access to initial quality treatment and later on to poorer survival.

Table 3. Recommendations for adjuvant therapy for women 70 years old or older*

Risk category	Size, ER status, grade	Treatment
Lymph node negative, low (<10%)	≤1 cm, nonpalpable	None or Tam
Lymph node negative, intermediate (10%–15%)	>1 ≤2 cm, ER+, grade I or II	Tam ± Chemo
Lymph node negative, high (>15%)	≥1 cm and ER– or >2 cm and ER+ or grade III	Tam and/or Chemo if tolerated
Lymph node positive	ER+ or PR+ ER– and PR–	Tam Chemo

*From Goldhirsch et al. (22). ER = estrogen receptor; Tam = tamoxifen; Chemo = chemotherapy.

Table 4. National Institute of Aging (NIA) and National Cancer Institute (NCI) program announcements related to cancer and aging

Announcement No.	Title
PA 96-034	Aging, Women and Breast Cancer (NIA/NCI)
PA 98-069	Cancer Pharmacology and Treatment in Older Patients (NIA/NCI)
PA 99-030	Aging and Old Age as Risk Factors for Multiple Primary Tumors (NIA)
October 1998	Studies on Older Patients in the NCI Clinical Trials Cooperative Groups (NIA/NCI)

Efforts are needed to provide all Americans with access to quality medical care. The survival benefits of systemic adjuvant therapy in early breast cancer are undisputed, and these treatments should be available to all Americans. Research is needed to determine the molecular mechanisms that underlie the differences in tumor biology among diverse racial and ethnic groups. For older Americans, research is needed on the interactions of adjuvant therapy and coexisting illness and how such interactions affect quality of life, activities of daily living, and survival. Moreover, education of physicians and the public concerning issues related to cancer in older patients is necessary to overcome age bias in treatment selection.

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NOTE

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Duration of Adjuvant Tamoxifen Therapy

John Bryant, Bernard Fisher, James Dignam

The benefit of using adjuvant tamoxifen to treat breast cancer has been firmly established for patients with estrogen receptor (ER)-positive tumors, regardless of age, lymph node status, or menopausal status. Uncertainty remains, however, regarding the optimal duration of tamoxifen therapy. We reviewed the findings of randomized clinical trials that directly compared alternative treatment durations. Trials comparing short-term adjuvant treatment with tamoxifen (i.e., 1–3 years) with treatments having durations of about 5 years consistently have demonstrated additional benefits stemming from the longer therapy. Trials testing 5 years of treatment with longer durations have, in the aggregate, suggested no additional benefit for the patient. Nevertheless, the number of recurrences reported to date in these trials is not large, and the results of the individual trials are heterogeneous. Furthermore, as a result of tamoxifen's "carryover" effect, duration trials require considerable follow-up before definitive results can be established. Until more definitive data become available, adjuvant treatment with tamoxifen should be limited to 5 years outside the clinical trials setting. Continued accrual of ER-positive patients to ongoing tamoxifen duration trials, including the Adjuvant Tamoxifen Treatment Offer More (aTTom) and Adjuvant Tamoxifen Longer Against Shorter (ATLAS) trials, is appropriate. Alternatively, patients who remain disease free after 5 years of tamoxifen therapy should be encouraged to participate in trials testing crossover to other hormonal interventions, including selective ER modulators or aromatase inhibitors. [*J Natl Cancer Inst Monogr* 2001;30:56–61]

Clinical trials of adjuvant tamoxifen therapy have convincingly demonstrated benefit for patients with estrogen receptor (ER)-positive breast cancer, regardless of age, lymph node status, or menopausal status (1–3). Despite the large number of trials that have tested tamoxifen, however, uncertainty remains regarding the optimal duration of therapy. We will briefly review clinical trials comparing short-term administration of tamoxifen therapy (i.e., 1–3 years) with treatment durations of about 5 years, from which considerable evidence has accumulated in the last decade. We will be primarily concerned, however, with summarizing those trials that compare 5 years of treatment with longer durations. The data currently available from these trials are not extensive, and their interpretation is controversial.

DURATIONS OF UP TO 5 YEARS

Four trials have been reported in the past decade that compare 5 years of tamoxifen treatment with shorter durations of therapy. Aspects of the trials' designs and patient populations are summarized in Table 1. Two Eastern Cooperative Oncology Group (ECOG) trials, E4181 (4) and E5181 (5), compared 1 year of tamoxifen therapy with 5 years of the same therapy in postmenopausal and premenopausal lymph node-positive patients, respectively. Patients in both trials received adjuvant chemotherapy in

addition to tamoxifen. The Swedish Breast Cancer Cooperative Group (Swedish BCCG) (6) trial was a larger study that compared 2 years of tamoxifen therapy with 5 years of the same therapy in 3545 postmenopausal patients, only 2.5% of whom received chemotherapy. The Cancer Research Campaign (CRC) (7) trial also compared 2 years of tamoxifen therapy with 5 years of tamoxifen therapy in 2937 patients aged 50 years or older. In addition to tamoxifen, patients in this trial were treated with adjuvant chemotherapy or not, according to local protocols. The results of a fifth study, TAM-01 (8), were first reported in 2000. This trial is designed to compare 2–3 years of tamoxifen therapy with 12–13 years of the same therapy, but the median duration of tamoxifen therapy at the time of the report was 30 months on the control arm and 70 months on the extended treatment arm, so the currently available data may be interpreted as comparing 2–3 years of treatment with approximately 6 years of treatment. About 30% of the 3793 patients included in this study also received adjuvant chemotherapy. Both ER-negative and ER-positive patients were included in all five trials, in the proportions indicated in Table 1.

Each of these five studies has reported statistically significant reductions in event rates in favor of the longer duration of treatment. The three studies that compared a treatment duration of about 2 years with treatment lasting at least 5 years [Swedish BCCG (6), CRC (7), and TAM-01 (8)] all estimated a proportional reduction in event rate of about 20%. Only one of the five studies [Swedish BCCG (6)] reported a statistically significant survival advantage for the longer duration (18% proportional reduction in mortality rate; 95% confidence interval [CI] = 1% to 31%; $P = .03$). One additional trial (CRC) (7) also showed a modest but nonsignificant survival advantage (11% reduction in mortality rate; 95% CI = –15% to 31%). In light of the strong evidence for a reduced recurrence rate, however, it is reasonable to attribute this to the relatively short follow-up reported and to the well-known carryover effect of treatment with tamoxifen (1,3). A preliminary meta-analysis of direct comparisons of short-term tamoxifen therapy with those lasting about 5 years, which was presented for discussion at the September 21–23, 2000, Oxford meeting of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), did in fact indicate a modest but statistically significant mortality advantage for the longer duration. Published indirect comparisons of trials of about 1, 2, or 5 years' duration based on the 1998 EBCTCG overview led to a similar conclusion (1), as trends for both reduced recurrence rate and mortality over this range were statistically significant.

Affiliations of authors: J. Bryant, National Surgical Adjuvant Breast and Bowel Project (NSABP) Biostatistical Center, Pittsburgh, PA, and Department of Biostatistics, University of Pittsburgh; B. Fisher, NSABP and Department of Surgery, University of Pittsburgh; J. Dignam, NSABP Biostatistical Center and Department of Health Studies, University of Chicago, IL.

Correspondence to: John Bryant, Ph.D., NSABP Biostatistical Center, 1 Sterling Plaza, 230 N. Craig St., Suite 350, Pittsburgh, PA 15213 (e-mail: bryant@nsabp.pitt.edu).

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Table 1. Reports comparing 1–2 years of tamoxifen therapy with about 5 years*

Study	Investigator, y (reference No.)	Duration comparison	Patient population	ER status	Adjuvant chemotherapy	No. of analyzed patients	Median follow-up, y†
ECOG E4181	Falkson et al., 1990 (4)	1 y vs. 5 y; 10 mg b.i.d.	Postmenopausal, lymph node +	70% ER+, 30% ER–	Yes	542	3.1
ECOG E5181	Tormey et al., 1992 (5)	1 y vs. 5 y; 10 mg b.i.d.	Premenopausal, lymph node +	65% ER+, 35% ER–	Yes	396	4.1
Swedish	Swedish BCCG, 1996 (6)	2 y vs. 5 y; 20 or 40 mg/day	Postmenopausal, lymph node +/-	60% ER+, 16% ER–, 23% ER?	2.5% received chemotherapy	3545	3.5
CRC	CRC, 1996 (7)	2 y vs. 5 y; 20 mg/day	≥50 y old, lymph node +/-	NR‡	Permitted per local protocols	2937	2.0
TAM-01	Delozier et al., 2000 (8)	2–3 y vs. 6 y§; 20, 30, or 40 mg/day	Pre/postmenopausal, lymph node +/-	64% ER+, 9% ER–, 26% ER?	30% received chemotherapy	3793	4.0

*ECOG = Eastern Cooperative Oncology Group; ER = estrogen receptor; CRC = Cancer Research Campaign; BCCG = Breast Cancer Cooperative Group;

– = negative; + = positive; b.i.d. = twice a day.

†Median follow-up beyond point at which tamoxifen therapy was either to be stopped or to be continued.

‡NR = not reported.

§The trial is designed to compare 2–3 years of treatment with 12–13 years, but the median duration of tamoxifen treatment at the time of the report was 30 months in the short-term group and 70 months in the long-term group.

||Three patients received 10 mg/day, and one patient received 1 mg/day.

TREATMENT FOR 5 YEARS VERSUS LONGER DURATION

Findings from three trials that compared 5 years of tamoxifen therapy with treatments of longer duration were first reported in 1996. Aspects of the trials' designs and patient populations are summarized in Table 2. These trials included National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 (3), a double-blind comparison of 5 additional years of tamoxifen therapy versus placebo in 1172 lymph node-negative women who were disease free after completing 5 years of initial treatment with tamoxifen; the Scottish trial (9), in which 342 predominantly lymph node-negative women were assigned to receive indefinite tamoxifen therapy or to observation following the completion of 5 years of tamoxifen therapy disease-free; and ECOG E4181/E5181 (10), in which 87 postmenopausal lymph node-positive women from E4181 and 106 premenopausal lymph node-positive women from E5181 who had been treated with chemotherapy and 5 years of tamoxifen therapy and who were disease free at 5 years were randomly assigned to either indefinitely continued tamoxifen therapy or to observation. All patients in B-14 (3) were ER positive (≥10 fmol/mg protein),

compared with 73% in the ECOG trial (10). In the Scottish trial (9), 39% of patients had tumors with ER content of 20 fmol/mg or more, 22% had tumors with ER content of 19 fmol/mg or less, and 39% had tumors that were not assayed. Updated data from the NSABP study (3) and the Scottish trial (9) have been published recently (11,12).

National Surgical Adjuvant Breast and Bowel Project B-14

After a median follow-up of 6.75 years from random assignment to either placebo or continued tamoxifen, the B-14 data demonstrated no advantage for continued therapy, and in fact the trend moved in the opposite direction. Seven years following randomization, the overall survival rate was 94% for placebo-treated patients and 91% for tamoxifen-treated patients (39 versus 57 deaths, respectively; hazard ratio [HR] = 1.5 [95% CI = 1.0 to 2.2]; $P = .07$). Disease-free survival (DFS) rate was 82% for placebo patients and 78% for those who received more than 5 years of tamoxifen therapy (106 versus 137 events; HR = 1.3 [95% CI = 1.0 to 1.7]; $P = .03$).

Table 2. Published reports comparing 5 years of tamoxifen therapy with more than 5 years of therapy*

Study	Investigator, y (reference No.)	Duration comparison	Patient population	% ER+, ≥10 fmol/mg	Adjuvant chemotherapy	No. of analyzed patients	Median follow-up, y†
NSABP B-14	Fisher et al., 1996 (3)	5 y vs. 10 y;‡ 10 mg b.i.d.	Pre/postmenopausal, ER+, lymph node–	100% ER+	No	1152§	6.75 (at 2 nd report)
Scottish trial	Fisher et al., 2001 (11) Stewart et al., 1996 (9) Stewart et al., 2001 (12)	5 y vs. indefinite; 20 mg/day	Premenopausal, lymph node –; or postmeno- pausal, lymph node +/- (70% negative, 23% positive, and 7% status unknown)	39% ≥20 fmol/mg, 22% <20 fmol/mg, 39% ?	No	342	10 (at 2 nd report)
ECOG E4181/E5181	Tormey et al., 1996 (10)	5 y vs. indefinite; 10 mg b.i.d.	Pre/postmenopausal, lymph node +	73% ER+, 27% ER–	Yes	193	5.6

*NSABP = National Surgical Adjuvant Breast and Bowel Project; ER = estrogen receptor; ECOG = Eastern Cooperative Oncology Group; – = negative; + = positive; b.i.d. = twice a day.

†Median follow-up beyond randomization to either stop or continue tamoxifen therapy after 5 years of treatment.

‡Treatment was terminated before all patients had completed therapy. Mean additional tamoxifen therapy received following randomization was 38 months.

§The reported analysis was based on the eligible cohort, consisting of 1152 of 1172 total patients randomly assigned.

Events in the primary NSABP DFS analysis included recurrence, contralateral breast cancer (CBC), other second primary cancers, and death (11). The distribution of sites of first events is given in Table 3. Only about one half (118 of 243) of all first events were breast cancer related (i.e., recurrences or CBC). For comparison with other datasets, we performed an analysis of breast cancer first events only; the pattern is similar to that seen for DFS, but the suggestion of detriment is not as strong. Of the 118 recurrences or CBCs on both arms, 54 occurred among control patients, and 64 occurred among those who continued to take tamoxifen. This corresponds to a 21% proportional increase in event rate, which is not statistically significant ($P = .31$).

Table 3 also shows a twofold increase in the incidence of endometrial cancers (six versus 12) in women assigned to the longer therapy duration. This is consistent with the well-documented increase in risk for endometrial cancer in women using tamoxifen (1,13,14). Despite some (nonsignificant) excess seen in Table 3 for other second cancers and deaths before treatment failure among patients who received continued tamoxifen therapy, no other particular site of second primary cancer or cause of death was noticeably imbalanced between the two treatment arms (11).

It may be expected that the efficacy of continued tamoxifen therapy, relative to patients randomly assigned to receive placebo after an initial 5 years of tamoxifen therapy, will not remain constant over time. If the carryover effect of the initial tamoxifen on the outcome of patients assigned to receive placebo diminishes slowly over time, the relative benefit of continued treatment would be small in the initial period following randomization but greater later on as the effect of prerandomization therapy attenuates. Therefore, it is useful to look at the data after events have been categorized according to whether they occurred within years 5–10 from the initial surgical treatment (i.e., in the first 5 years after the divergence of treatments) or whether they occurred subsequent to that time. Table 4 summarizes the occurrence of first events that compose the DFS end point, while Table 5 shows the pattern for all-cause mortality. From Table 4 it is seen that, in the first 5 years after the divergence of treatments, the rate of occurrence of all DFS events among patients assigned to tamoxifen therapy was about 60% greater than that among patients who were randomly assigned to receive placebo ($HR = 1.6$ [95% CI = 1.1 to 2.2]; $P = .007$), but in the second 5 years, the relative risk was equal to 1.0. (A very similar pattern is seen for mortality in Table 5.) For breast cancer-related events (recurrences and CBCs), there is a statistically significant excess of events (48 versus 27 events; $P = .02$) in the first 5-year period among patients who received continued tamoxifen

therapy. This pattern is not repeated in the second 5 years, and in fact, there is a nonsignificant net benefit of 11 events (16 versus 27 events; $P = .13$).

It is not clear how to interpret this pattern, which may be a result of chance. However, it is not easy to ascribe the suggestion of a "turnaround" in the data to a carryover effect, which would be more consistent with a pattern in which there was limited early benefit for continued treatment, coupled with greater benefit later on. The observed pattern could be consistent either with the phenomenon of tamoxifen withdrawal response or with the arrested growth of tumors that have become tamoxifen dependent, resulting in a temporary delay in recurrence following withdrawal of tamoxifen; but, of course, this is speculative.

Scottish Trial

The initial report of findings from the Scottish trial (9) concluded, after 6.2 years of follow-up, that a meaningful benefit from continuing adjuvant tamoxifen therapy beyond 5 years was unlikely. A recently published update of these data (12) (median follow-up, about 10 years) continues to show the same general pattern (i.e., those patients receiving >5 years of tamoxifen therapy had higher rates of recurrence and death than did those whose treatment stopped at 5 years), although the differences do not achieve statistical significance. Of the 169 patients randomly assigned to stop tamoxifen therapy after 5 years, 38 have had breast cancer events (recurrences or CBCs) subsequent to randomization, compared with 49 of the 173 patients assigned to continued treatment (Table 6). Similarly, there have been 16 more deaths from all causes among patients assigned to longer treatment (70 versus 54; $HR = 1.32$ [95% CI = 0.93 to 1.88]; $P = .12$). Comparable results were obtained in analyses of deaths from breast cancer ($HR = 1.44$ [95% CI = 0.87 to 2.38]; $P = .15$) and survival free of systemic disease, including CBCs ($HR = 1.36$ [95% CI = 0.95 to 1.95]; $P = .12$). Even when the analysis was restricted to only those patients having strong ER expression (≥ 20 fmol/mg), the rates of breast cancer events, all-cause mortality, and breast cancer mortality did not differ statistically across study arms. As was the case in NSABP B-14, there were more endometrial cancers among patients randomly assigned to indefinite tamoxifen therapy than among those who stopped the drug after 5 years (six versus two, respectively).

Published Findings From ECOG E4181/E5181

In contrast to both NSABP B-14 (3) and the Scottish trial (9), published results from ECOG E4181/E5181 are more suggestive of a possible benefit for continuing treatment with tamoxifen

Table 3. B-14: 5-year randomization to placebo or continued tamoxifen therapy, sites and rates of first events

	Placebo (n = 569*)			Tamoxifen (n = 583*)			Placebo vs. tamoxifen		
	No. of events	%	Rate†	No. of events	%	Rate†	Hazard ratio	95% CI‡	P
Breast cancer recurrences	34	6.0	8.9	47	8.1	12.5	1.4	0.9 to 2.2	
Contralateral breast cancers	20	3.5	5.2	17	2.9	4.5	0.9	0.4 to 1.7	
Endometrial cancers	6	1.1	1.6	12	2.1	3.2	2.0	0.7 to 6.6	
Other second primary cancers	28	4.9	7.3	34	5.8	9.0	1.2	0.7 to 2.1	
Deaths prior to recurrence or second primary cancer	18	3.2	4.7	27	4.6	7.2	1.5	0.8 to 2.9	
All events	106	18.6	27.6	137	23.5	36.3	1.3	1.0 to 1.7	.03

*Analysis was based on the cohort of eligible patients (n = 1152). Similar results were obtained when all randomly assigned patients were included.

†Average annual rate per 1000 patients.

‡CI = confidence interval.

Table 4. B-14: sites and rates of first events occurring within 5 years after randomization and thereafter

Time interval	Event	Placebo (n = 569 [‡])		Tamoxifen (n = 583 [‡])		Placebo vs. tamoxifen		
		No. of events	Rate [†]	No. of events	Rate [†]	Hazard ratio	95% CI [‡]	P
Within 5 y of randomization to placebo or continued tamoxifen therapy	Breast cancer recurrences	18	6.7	36	13.5	2.0	1.1 to 3.8	
	Contralateral breast cancers	9	3.3	12	4.5	1.3	0.5 to 3.6	
	Endometrial cancers	4	1.5	8	3.0	2.0	0.5 to 9.1	
	Other second primary cancers	18	6.7	21	7.9	1.2	0.6 to 2.3	
	Deaths prior to recurrence or second primary cancer	10	3.7	15	5.6	1.5	0.6 to 3.8	
	All events in first 5 y following randomization	59	22.0	92	34.4	1.6	1.1 to 2.2	.007
>5 y from randomization to placebo or continued tamoxifen therapy	Breast cancer recurrences	16	13.9	11	10.0	0.7	0.3 to 1.6	
	Contralateral breast cancers	11	9.6	5	4.5	0.5	0.1 to 1.5	
	Endometrial cancers	2	1.7	4	3.6	2.1	0.3 to 23	
	Other second primary cancers	10	8.7	13	11.8	1.4	0.6 to 3.4	
	Deaths prior to recurrence or second primary cancer	8	7.0	12	10.9	1.6	0.6 to 4.4	
	All events occurring subsequent to 5 years	47	41.0	45	40.8	1.0	0.7 to 1.5	.96

*Analysis was based on the cohort of eligible patients (n = 1152). Similar results were obtained when all randomly assigned patients were included.

†Average annual rate per 1000 patients.

‡CI = confidence interval.

Table 5. B-14: deaths occurring within first 5 years after randomization and thereafter

	Placebo (n = 569 [‡])		Tamoxifen (n = 583 [‡])		Placebo vs. tamoxifen		
	No. of deaths	Rate [†]	No. of deaths	Rate [†]	Hazard ratio	95% CI [‡]	P
Deaths occurring within first 5 y after randomization to placebo or continued tamoxifen therapy	20	7.2	35	12.4	1.7	1.0 to 3.2	.051
Deaths occurring >5 y after randomization to placebo or continued tamoxifen therapy	19	14.9	22	17.4	1.2	0.6 to 2.3	.61
All deaths	39	9.6	57	14.0	1.5	1.0 to 2.2	.07

*Analysis was based on the cohort of eligible patients (n = 1152). Similar results were obtained when all randomly assigned patients were included.

†Average annual rate per 1000 patients.

‡CI = confidence interval.

Table 6. Overview of trials comparing about 5 years of tamoxifen therapy with longer durations*

	Frequency of patients having breast cancer events, i.e., recurrence or CBC/No. of analyzed patients (%)			Frequency of deaths from all causes/No. of analyzed patients (%)		
	Shorter duration of tamoxifen therapy	Longer duration of tamoxifen therapy	Total frequency	Shorter duration of tamoxifen therapy	Longer duration of tamoxifen therapy	Total frequency
NSABP B-14	54/569 (9.5)	64/583 (11)	118/1152 (10)	39/569 (6.9)	57/583 (9.8)	96/1152 (8.3)
Scottish trial	38/169 (22)	49/173 (28)	87/342 (25)	54/169 (32)	70/173 (40)	124/132 (36)
ECOG E4181/E5181	29/93 (31)	17/100 (17)	46/193 (24)	23/93 (25)	22/100 (22)	45/193 (23)

*CBC = contralateral breast cancer; NSABP = National Surgical Adjuvant Breast and Bowel Project; ECOG = Eastern Cooperative Oncology Group.

beyond 5 years (10). After a median follow-up of 5.6 years following randomization at 5 years, a nonsignificant advantage was seen for extended tamoxifen therapy in terms of time to first recurrence or CBC (23 versus 15 breast cancer events; recurrence-free survival at 5 years after randomization = 73% for those stopping therapy at 5 years versus 85% for those continuing tamoxifen therapy; $P = .10$). Similar results were obtained in an analysis of DFS (defined in this analysis as the time from the randomization to first recurrence, CBC, or death without a prior breast cancer event). Of note, in a secondary analysis restricted to those patients who were ER positive (≥ 10 fmol/mg), the comparison of times to recurrence or CBC became statistically significant (22 versus 12 events; $P = .014$). There was, however, no evidence of any survival benefit for continued tamoxifen therapy (10 deaths in patients who received 5 years of

treatment versus 14 among patients who received extended tamoxifen therapy, $P = .52$).

Updated Data From ECOG E4181/E5181

Unpublished updated analyses of the ECOG E4181/E5181 data were provided by the ECOG statistical office for discussion in this review (Gray R: personal communication). These data, which provide about 4 years of extended follow-up beyond the published findings, are summarized in Table 6. They continue to show a trend toward benefit for extended treatment. In an analysis of time to recurrence or CBC, the difference in breast cancer event rates between the two arms was statistically significant: 29 of 93 patients randomly assigned to stop tamoxifen therapy at 5 years experienced breast cancer recurrences or CBCs, compared with 17 of 100 patients randomly assigned to receive

indefinite tamoxifen therapy (HR = 0.53; $P = .03$). A comparison of DFS between the two study arms was not statistically significant but was suggestive of some benefit for continued treatment (HR = 0.68; $P = .13$). However, the updated data still failed to demonstrate evidence of benefit in terms of overall mortality (23 deaths among patients who received 5 years of tamoxifen therapy, compared with 22 deaths among those assigned to receive indefinite tamoxifen therapy). Three endometrial cancers have been reported in patients who received extended tamoxifen therapy, while one has been reported in the group that stopped treatment after 5 years.

Preliminary Data From Ongoing Duration Trials

Both the Adjuvant Tamoxifen Treatment Offer More (aTTom) (15) and the Adjuvant Tamoxifen Longer Against Shorter (ATLAS) (16) trials continue to accrue patients with primary breast cancer who have received initial adjuvant treatment with tamoxifen and who are randomly assigned to either continue receiving the drug or to observation. Current accrual is essentially limited to patients who have received about 5 years of initial treatment (Peto R: personal communication). These trials are considerably larger than previous comparisons of 5 years' duration to longer treatment duration and should, over the next decade, provide definitive answers to the duration question.

At the recent Oxford EBCTCG meeting (September 21–23, 2000), unpublished data from 6779 aTTom and ATLAS patients, all of whom had been randomly assigned after completing—disease-free—at least 4 years of tamoxifen therapy, were combined in a blinded fashion with data from NSABP B-14 (3), the Scottish trial (9), and ECOG E4181/E5181 (10) to provide a preliminary meta-analysis of the tamoxifen duration question. Available follow-up of the aTTom and ATLAS patients was very short, with a median follow-up of probably no more than 1 year and virtually no follow-up beyond 5 years after randomization. Nevertheless, these patients had experienced 195 breast cancer events and 128 deaths, thereby adding substantially to the number of events observed in the earlier trials. The combined data resulted in estimated HRs for both breast cancer events and mortality that were not statistically different from 1. The combined data also provided a (statistically nonsignificant) suggestion of benefit in the second 5-year period following the divergence of treatments. However, almost all of the data supporting this trend came from NSABP B-14 (3), as is illustrated in Table 7. As has been discussed previously, the time-dependent pattern of recurrence seen in the B-14 trial is not readily explained by the carryover phenomenon.

DISCUSSION

Randomized clinical trials of adjuvant tamoxifen therapy have overwhelmingly demonstrated its efficacy for patients with

ER-positive breast cancer, without regard to age, lymph node status, or menopausal status at random assignment (1–3). In addition, within the last decade, sufficient evidence has been obtained to firmly conclude that 5 years of treatment is superior to short-term treatment of about 2 years (4–8). The advisability of continuing treatment beyond 5 years has not been established (3,9,10–12).

In the aggregate, data from published clinical trials comparing 5 years of tamoxifen therapy with more than 5 years of tamoxifen therapy are disappointing and do not demonstrate any benefit from extended treatment (Table 6). However, there are good reasons to withhold judgment on the duration question until considerably more data are available. First, the number of breast cancer events (recurrences and CBCs) and deaths observed to date in the published trials [NSABP B-14 (3), Scottish trial (9), and ECOG E4181/E5181 (10)] is considerably smaller (251 breast cancer events and 265 deaths) than is required to achieve consensus. This remains the case even if currently available preliminary data are included from the ongoing aTTom (15) and ATLAS (16) trials. Second, it has been firmly established that treatment with tamoxifen conveys a considerable carryover benefit to the patient that lasts some years after the termination of treatment. For this reason, one might expect the effectiveness of extended tamoxifen therapy (compared with therapy of just 5 years' duration) to be partially attenuated in the initial period following the divergence of treatments; indeed, it may not become apparent until extended follow-up has been obtained. Finally, the results of published trials comparing 5 years of tamoxifen therapy with treatments of longer duration are heterogeneous: Both the NSABP B-14 trial (3) and the Scottish trial (9) show no benefit and, in fact, are weakly suggestive of some detriment. The ECOG E4181/E5181 (10) trial, however, while smaller than the other two trials, is supportive of the hypothesis of continued benefit.

The apparent difference in the results of the ECOG trial (10) and the other two studies could be the result of chance, although a heterogeneity test for between-trial differences based on breast cancer events is marginally significant ($P = .025$). Alternatively, it has been suggested that the optimal duration of treatment might differ for lymph node-negative and lymph node-positive patients (10). The proportional reduction in risk afforded by extended tamoxifen therapy would probably not be expected *a priori* to be strongly dependent on lymph node status, since extensive overview data indicate that the proportional reduction in risk resulting from treatment with tamoxifen, in contrast to no hormonal treatment, does not differ greatly between lymph node-negative and lymph node-positive patients (1). Nevertheless, even if the proportional risk reduction associated with extended treatment relative to treatment of only 5 years were independent of lymph node status, this would not be the case for

Table 7. Breast cancer events occurring within first 5 years after randomization and thereafter*

	Frequency of patients having breast cancer events, i.e., recurrence or CBC, within 5 y of randomization		Frequency of patients having breast cancer events, i.e., recurrence or CBC, >5 y after randomization	
	Shorter duration of tamoxifen therapy	Longer duration of tamoxifen therapy	Shorter duration of tamoxifen therapy	Longer duration of tamoxifen therapy
NSABP B-14	27	48	27	16
Scottish trial	25	37	13	12
ECOG E4181/E5181	25	14	4	3

*NSABP = National Surgical Adjuvant Breast and Bowel Project; ECOG = Eastern Cooperative Oncology Group; CBC = contralateral breast cancer.

absolute risk, since the recurrence rate in node-negative patients is considerably less than that of node-positive patients, even after 5 disease-free years (17). Treatment with tamoxifen is associated with certain serious risks, most notably endometrial cancer, thromboembolic disease, and possibly stroke (1,2,11,13,14). Therefore, even if it could be demonstrated that continued tamoxifen therapy were efficacious, its risk/benefit ratio for lymph node-negative women would be higher than for lymph node-positive women (and higher for older women compared with younger women).

CONCLUSIONS AND RECOMMENDATIONS

Strong evidence exists that 5 years of tamoxifen therapy is superior to 2–3 years of therapy, but as yet, there is no convincing evidence that extending treatment beyond 5 years provides additional benefit. Outside the clinical trials setting, adjuvant treatment with tamoxifen should, therefore, be limited to 5 years' duration until such time as additional data become available to definitively resolve the issue.

Because currently available evidence is insufficient to achieve consensus regarding the benefit of treatment with tamoxifen beyond 5 years, continued accrual of patients with hormone-responsive tumors from whom proper consent has been obtained to long-term tamoxifen trials, including aTTom and ATLAS, is appropriate. Alternatively, patients who have remained disease free for extended periods of time on tamoxifen therapy should strongly consider participation in clinical trials of other hormonal interventions, including aromatase inhibitors or selective ER modulators. Currently, crossover of hormone-responsive postmenopausal patients to aromatase inhibitors following 5 disease-free years of tamoxifen therapy is being studied in at least three trials, including National Cancer Institute of Canada Study MA 17 (letrozole) (18), NSABP B-33 (exemestane) (17), and Austrian Breast Cancer Study Group Study 6A (anastrozole) (18).

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Findings From Recent National Surgical Adjuvant Breast and Bowel Project Adjuvant Studies in Stage I Breast Cancer

Bernard Fisher, Jong-Hyeon Jeong, James Dignam, Stewart Anderson, Eleftherios Mamounas, D. Lawrence Wickerham, Norman Wolmark

Before 1989, credible information about the treatment of breast cancer was derived mainly from randomized clinical trials that enrolled women with either metastatic (stage IV); locally advanced (stage III); or primary, operable, axillary lymph node-positive (stage II) disease. This report provides information from six recent National Surgical Adjuvant Breast and Bowel Project (NSABP) trials involving lymph node-negative (stage I) patients. Findings from NSABP B-13 demonstrated, through 14 years of follow-up, improvements in disease-free survival (DFS) and overall survival from methotrexate and fluorouracil (MF), regardless of age, in women with estrogen receptor (ER)-negative tumors. Results from NSABP B-19, which was conducted with similar patients, demonstrated, through 8 years, a greater overall DFS and survival advantage with cyclophosphamide and MF (CMF) than that observed with MF. Findings from NSABP B-23, in which patients similar to those in B-13 and B-19 were randomly assigned to receive CMF plus placebo, CMF plus tamoxifen (TAM), doxorubicin (Adriamycin) and cyclophosphamide (AC) plus placebo, or AC plus TAM, demonstrated no difference in relapse-free survival (RFS) or overall survival among the four groups through 5 years, either for all patients or relative to age. NSABP B-14, which was carried out in women with ER-positive tumors, compared the outcomes of those who received either placebo or TAM. Through 14 years, superior DFS and overall survival advantages, as well as a reduction in contralateral breast cancer, were observed with TAM. No additional benefit resulted from TAM administration beyond 5 years. Findings from NSABP B-20, a second study conducted in patients with ER-positive tumors, showed, after 8 years, both a DFS and an overall survival advantage from TAM plus either MF or CMF over that achieved with TAM alone. A recent meta-analysis in women with negative lymph nodes and either ER-negative or ER-positive tumors of less than or equal to 1 cm in size was conducted using patients from five NSABP trials. After 8 years, the RFS in women with ER-negative tumors was greater in the group treated with surgery and chemotherapy than in those who underwent surgery alone. In women with ER-positive tumors, RFS and overall survival advantages were observed from the addition of chemotherapy to TAM when that treatment regimen was compared with TAM alone. In addition, evidence has been presented from NSABP B-21, a trial evaluating radiation therapy (XRT) and/or TAM for the prevention of ipsilateral breast tumor recurrence (IBTR) after lumpectomy in women with tumors less than or equal to 1 cm. Findings have shown that XRT is superior to TAM and that XRT + TAM is superior to XRT alone for preventing IBTR. The findings demonstrate that chemotherapy and/or hormonal therapy is

effective for the management of women with negative axillary lymph nodes and either ER-negative or ER-positive tumors. Because it also has been proven effective in women with tumors less than or equal to 1 cm, such therapy might also be considered in the treatment of that patient population. [J Natl Cancer Inst Monogr 2001;30:62-6]

INTRODUCTION

At a National Institutes of Health (NIH) consensus conference convened in 1985, data on adjuvant chemotherapy and endocrine therapy that had been obtained from randomized trials conducted during the 1970s and early 1980s were evaluated. It was concluded that there was inadequate information to justify recommending therapy other than surgery for women with negative axillary lymph nodes (1). By 1990, however, findings from studies conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP) and by other investigators warranted a second conference on the treatment of early-stage breast cancer. Meeting participants concluded that "the rate of local and distant recurrence following local therapy for lymph node-negative breast cancer is decreased by both adjuvant combination of cytotoxic chemotherapy and by adjuvant tamoxifen" (2). It was also noted that patients with tumors of less than or equal to 1 cm had an excellent prognosis and would not require adjuvant systemic therapy outside of clinical trials. Participants acknowledged, however, that the completed studies were not large enough, nor the follow-up long enough, to allow for an accurate estimation of the interactions between menopausal status or steroid receptor positivity and the effects of adjuvant therapy in lymph node-negative patients. They also noted that there were too few patients with estrogen receptor-negative tumors to permit an evaluation of the benefit of tamoxifen, that the follow-up was too short for meaningful mortality data to be obtained, and that not enough information was available to permit a determination of the effects of combination chemotherapy plus tamoxifen in patients with negative lymph nodes. Since the 1990 conference, findings from six NSABP trials using 11 620 patients with primary (stage I) breast cancer and negative axillary lymph

Affiliations of authors: B. Fisher, D. L. Wickerham, N. Wolmark, National Surgical Adjuvant Breast and Bowel Project (NSABP), Pittsburgh, PA; J.-H. Jeong, S. Anderson, Department of Biostatistics, University of Pittsburgh; J. Dignam, NSABP and Department of Health Studies, University of Chicago, IL; E. Mamounas, Cancer Center, Aultman Hospital, Canton, OH.

Correspondence to: Bernard Fisher, M.D., National Surgical Adjuvant Breast and Bowel Project, 4 Allegheny Center, Suite 602, Pittsburgh, PA 15212 (e-mail: bernard.fisher@nsabp.org).

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nodes have provided information to address these issues. Numerous reports of the results from these studies have either been published or are currently in press. Highlights of the most recent findings from the studies were presented at the November 2000 NIH consensus conference on adjuvant therapy for breast cancer. This report provides an overview of that presentation.

PATIENTS WITH ESTROGEN RECEPTOR-NEGATIVE TUMORS AND NEGATIVE AXILLARY LYMPH NODES

Worth of Adjuvant Chemotherapy

In the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-13 trial, women were randomly assigned to receive either surgery and no chemotherapy or surgery followed by 12 courses (1 year) of methotrexate and sequentially administered 5-fluorouracil (MF) followed by leucovorin. Findings through 14 years of follow-up demonstrated that the improvements in overall disease-free survival and survival from MF, previously reported after 5 (3) and 8 (4) years, have persisted ($P < .0001$ in the former and $P = .02$ in the latter; Fig. 1). A similarly statistically significant disease-free survival benefit was observed for women aged less than or equal to 49 years and for those aged greater than or equal to 50 years ($P = .005$ in the former and $P = .001$ in the latter). A slight but not statistically significant difference in overall survival ($P = .3$) was observed in women aged less than or equal to 49 years, while a statistically significant overall survival benefit was observed in those aged greater than or equal to 50 years ($P = .02$). There was no evidence of an interaction between treatment group and age (e.g., $P = .34$) for overall survival. The risk ratios (and 95% confidence intervals [CIs]) relative to overall survival were 0.82 (95% CI = 0.56 to 1.17) for women aged less than or equal to 49 years and 0.59 (95% CI = 0.38 to 0.92) for those 50 years of age or older.

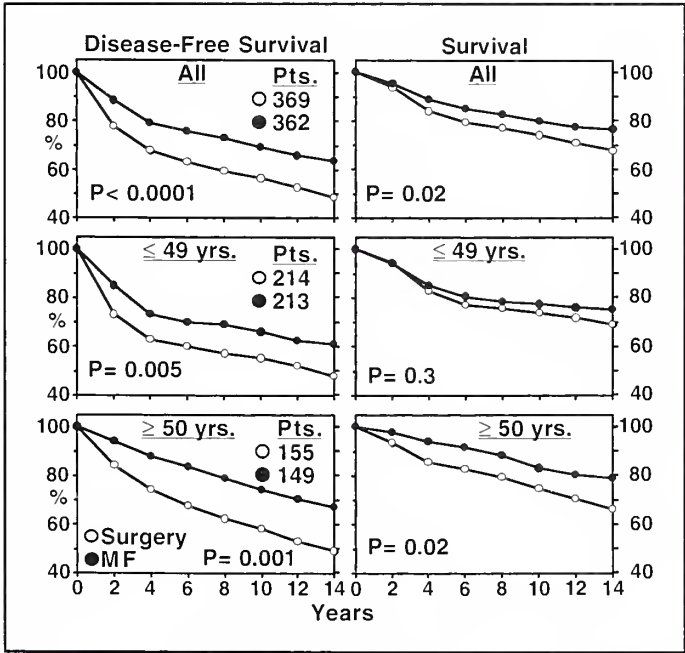


Fig. 1. Comparison of surgery with methotrexate and sequentially administered 5-fluorouracil (MF). Disease-free survival and overall survival in patients with estrogen receptor-negative tumors, according to treatment and age, National Surgical Adjuvant Breast and Bowel Project B-13. All *P* values were two-sided.

A second trial, NSABP B-19, was conducted to determine whether the alkylating agent cyclophosphamide contributed an additional benefit when administered with MF. Over a 6-month period, patients received either six courses of MF, as given in B-13, or six courses of cyclophosphamide and MF (CMF), as is conventionally used to treat breast cancer. Through 8 years, just as first reported after 5 years (4), greater overall disease-free survival and survival advantages ($P = .0003$ and $P = .03$, respectively) were obtained from treatment with CMF (Fig. 2). Those advantages were most evident in women aged less than or equal to 49 years ($P = .0004$ and $P = .007$, respectively). In women aged greater than or equal to 50 years, there was a small but nonsignificant advantage in both disease-free survival ($P = .2$) and overall survival ($P = .8$). As was observed above with regard to the B-13 study, there was no evidence of a statistically significant interaction between treatment and age group relative to disease-free survival ($P = .22$) and overall survival ($P = .08$). For women aged less than or equal to 49 years, the risk ratio was 0.59 (CI = 0.44 to 0.87), and for those aged greater than or equal to 50 years, the risk ratio was 0.78 (CI = 0.56 to 1.1). Relative to overall survival, the risk ratio was 0.6 (CI = 0.42 to 0.87) for women aged less than or equal to 49 years and 0.96 (CI = 0.63 to 1.45) for those 50 years of age or older.

Worth of Chemotherapy Plus Tamoxifen

The NSABP initiated the B-23 study because of contradictory information about the propriety of using tamoxifen (TAM) with chemotherapy and because of uncertainty about the relative worth of doxorubicin (Adriamycin; Pharmacia Upjohn, Peapack, NJ) and cyclophosphamide (AC) and CMF for the treatment of such patients. Women ($n = 2008$) were randomly assigned to receive CMF plus placebo, CMF plus TAM, AC plus placebo, or

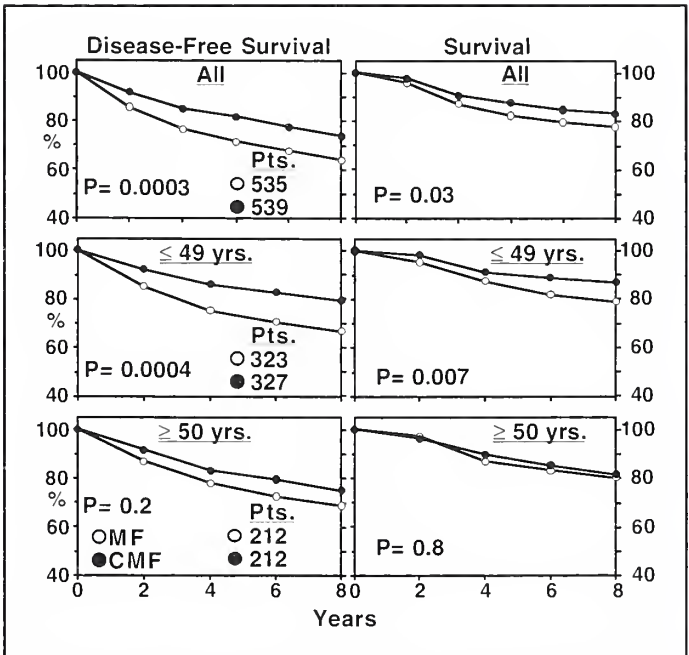


Fig. 2. Comparison of methotrexate and sequentially administered 5-fluorouracil (MF) with cyclophosphamide and MF (CMF). Disease-free survival and overall survival in patients with estrogen receptor-negative tumors, according to treatment and age, National Surgical Adjuvant Breast and Bowel Project B-19. All *P* values were two-sided.

AC plus TAM. Six cycles of CMF were given over 6 months; four cycles of AC were administered over 63 days. A first report of findings from that study (5) demonstrated (Fig. 3) no statistically significant difference in either relapse-free survival or overall survival among the four groups through 5 years, either for all patients ($P = 1.0$ and $P = .8$, respectively) or for those aged less than or equal to 49 or greater than or equal to 50 years (data not shown). Because of the low incidence of contralateral breast cancer in all groups of lymph node-negative patients in the B-23 study (i.e., 3% through 5 years), it is not possible to estimate the efficacy of administering TAM to such patients for the express purpose of reducing the incidence of contralateral breast tumors.

PATIENTS WITH ESTROGEN RECEPTOR-POSITIVE TUMORS AND NEGATIVE AXILLARY LYMPH NODES

Worth of TAM

NSABP B-14, a randomized, double-blind, placebo-controlled trial initiated in 1982, involved more than 2800 randomly assigned and 1200 registered TAM-treated patients. The aim of that study was to determine the effectiveness of TAM in patients with negative axillary lymph nodes. A significant advantage from TAM was first reported in 1989 after 5 years of follow-up (6). The disease-free survival and overall survival benefits have persisted through 14 years of follow-up ($P < .001$ and $P = .002$, respectively; Fig. 4) overall, as well as for women less than or equal to 49 years of age ($P < .001$ and $P = .01$, respectively) and for those aged greater than or equal to 50 years ($P < .001$ and $P = .04$, respectively). TAM therapy was also associated with a statistically significant reduction in contralateral breast cancer (6,7), but no additional benefit was observed from the administration of TAM beyond 5 years (8,9).

Worth of Chemotherapy plus TAM

NSABP B-20, a study that involved more than 2300 women, was aimed at testing the hypothesis that the addition of either MF or CMF to TAM would result in a greater benefit than that which could be achieved with TAM alone. After 8 years, as was previously shown after 5 years of follow-up (10), there continue to be significant disease-free survival and overall survival advantages from the use of TAM plus chemotherapy (84% versus 77%, $P = .001$, for disease-free survival; 92% versus 88%,

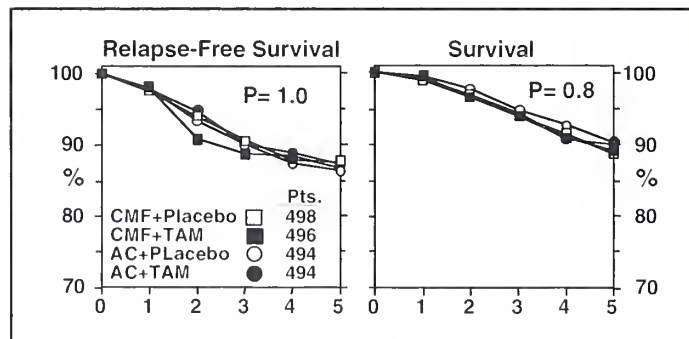


Fig. 3. Comparison of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) and placebo; CMF and tamoxifen (TAM); doxorubicin and cyclophosphamide (AC) and placebo; and AC and TAM. Relapse-free survival and overall survival in patients with estrogen receptor-negative tumors, according to treatment, National Surgical Adjuvant Breast and Bowel Project B-23. All P values were two-sided.

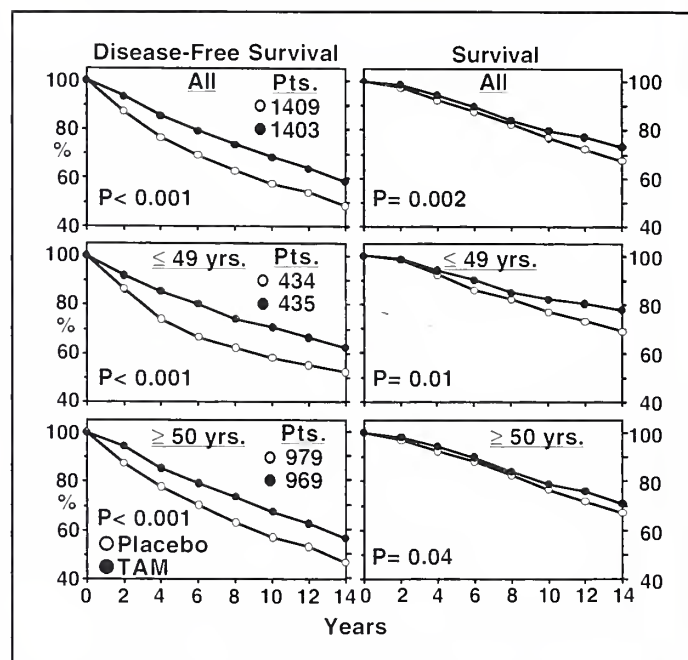


Fig. 4. Comparison of placebo with tamoxifen (TAM). Disease-free survival and overall survival in patients with estrogen receptor-positive tumors, according to treatment and age, National Surgical Adjuvant Breast and Bowel Project B-14. All P values were two-sided.

$P = .018$, for overall survival) (Fig. 5). That benefit was evident for both age groups, although more so for women aged less than or equal to 49 years than for those aged greater than or equal to 50 years.

WOMEN WITH ESTROGEN RECEPTOR-NEGATIVE OR ESTROGEN RECEPTOR-POSITIVE TUMORS OF LESS THAN OR EQUAL TO 1 CM

In an attempt to resolve uncertainty about the treatment and prognosis of patients with negative axillary lymph nodes and either estrogen receptor (ER)-negative or ER-positive tumors of less than or equal to 1 cm, data obtained from five NSABP randomized trials were obtained and analyzed collectively (11). The 8-year relapse-free survival rates for women with ER-negative tumors of less than or equal to 1 cm treated with surgery alone or with surgery and chemotherapy (MF or CMF)

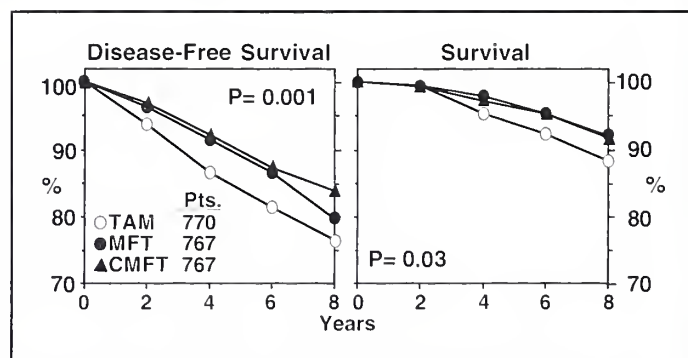


Fig. 5. Comparison of tamoxifen (TAM); methotrexate, 5-fluorouracil, and tamoxifen (MFT); and cyclophosphamide, methotrexate, 5-fluorouracil, and tamoxifen (CMFT). Disease-free survival and overall survival in patients with estrogen receptor-positive tumors, according to treatment, National Surgical Adjuvant Breast and Bowel Project B-20. All P values were two-sided.

were 81% and 90%, respectively ($P = .06$) (data not shown). Overall survival rates were similar in both groups (93% and 91%, respectively; $P = .65$). In women with ER-positive tumors of less than or equal to 1 cm who were treated with surgery alone, the 8-year relapse-free survival rate was 86%; the relapse-free survival rate was 93% after TAM was administered ($P = .01$) (data not shown) and 95% for women treated with TAM and chemotherapy (MF or CMF). When compared with the benefit achieved with the administration of TAM alone, the benefit achieved from the addition of chemotherapy to TAM approached statistical significance ($P = .07$). Overall survival rates among women in the three groups were 90%, 92% ($P = .41$), and 97% ($P = .01$), respectively.

RADIATION THERAPY AND/OR TAM FOR THE PREVENTION OF IPSILATERAL BREAST TUMOR RECURRENCE AFTER LUMPECTOMY IN WOMEN WITH TUMORS OF LESS THAN OR EQUAL TO 1 CM

Since the first report of results obtained from an NSABP trial (B-06) about the role of lumpectomy and breast irradiation for breast cancer, the question arose as to whether there were cohorts of women who did not require radiation therapy and whether such therapy might be replaced with TAM. NSABP B-21 was implemented to address that issue. Women with ER-positive or ER-negative tumors less than or equal to 1 cm ($n = 1009$) were randomly assigned to either TAM alone, radiation therapy plus placebo, or radiation therapy plus TAM. Recently reported results (12) have demonstrated a rate (per 1000 patients per year) of ipsilateral breast tumor recurrence (IBTR) of 23.3 for women who received TAM, 11.7 for those who received radiation therapy plus placebo, and 3.4 for those who received radiation therapy plus TAM.

SUMMARY AND COMMENTS

The findings presented in this report demonstrate a significant benefit from 1 year of MF therapy in patients with ER-negative tumors and negative axillary lymph nodes. The benefit from 6 months of MF therapy was not as good as that observed with 6 months of conventional CMF therapy. There was, however, no significant difference in outcome between women who received 6 months of CMF and those who received 2 months of AC. When TAM was given with either chemotherapy regimen, there was no significant advantage over that achieved from chemotherapy alone.

In women with ER-positive tumors and negative lymph nodes who received TAM therapy, a prolongation of disease-free survival and overall survival was present through 14 years of follow-up. Evidence of a reduction in IBTR and recurrences at other local and distant sites, as well as in tumor occurrence in the contralateral breast, persisted. These benefits were attained with a relatively low incidence of side effects. Study data demonstrate the worth of administering CMF with TAM to this cohort of patients.

These NSABP findings demonstrating the worth of adjuvant therapy for the treatment of patients with lymph node-negative breast cancer have been amply confirmed by the findings presented in the 1998 Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis (13). In its report, the EBCTCG noted, through 10 years, a 10.4% absolute benefit in relapse-free survival for patients under 50 years of age and a

5.7% absolute benefit in relapse-free survival in those patients aged 50–69 years, all of whom had received polychemotherapy. Similarly, a 5.7% and a 6.4% improvement in overall survival was noted in the two age groups, respectively, as a result of polychemotherapy.

The prognosis of women with ER-negative or ER-positive tumors of less than or equal to 1 cm and negative lymph nodes was somewhat better than that which has been reported for women with larger tumors. The prognosis was not, however, sufficiently favorable to dismiss the consideration of systemic therapy as an option for certain women with tumors of less than or equal to 1 cm. However, a cutoff point in the array of tumor sizes below 10 mm that would justify omission of systemic therapy remains to be established.

After lumpectomy for the treatment of women with tumors of less than or equal to 1 cm, the administration of radiation therapy and TAM prevents IBTR to a greater extent than does radiation therapy alone. TAM alone has been found to be inferior to radiation therapy in that regard.

Although findings from almost all of the studies have demonstrated a benefit, they have all engendered controversy with regard to their clinical application. As the prognosis of both treated and untreated cohorts of patients becomes better, therapeutic decision making becomes more difficult. The question arises as to what proportion of women in a cohort can be justifiably "written off" because it has been decided that the group's prognosis is sufficiently good to preclude treatment of any of the patients, despite evidence that some of them stand to benefit from a therapy of demonstrated efficacy. It would be inappropriate to deny women the opportunity to receive therapy from which they might benefit, because so many women with invasive cancer die each year despite having received "effective" treatment or because they have received either inadequate treatment or no treatment at all. In the 1990s, the death rate from breast cancer fell by 2%–3% per year. That circumstance was, in large part, a result of more widespread use of adjuvant therapy and more timely detection of the disease. It is likely that the judicious use of such therapy to treat women with smaller tumors and negative lymph nodes will result in increased overall survival among all women with breast cancer. It is, of course, necessary that extensive investigation be mandatory in an attempt to identify prognostic and predictive factors that provide justification for either administering or withholding a particular therapy from an individual patient. The NSABP has implemented a new generation of clinical trials to evaluate hypotheses that have been formulated as a result of the accumulation of new biologic information and the development of new agents that may improve the outcome of breast cancer patients via endocrine (hormonal) manipulation. One such study, NSABP B-33, is evaluating the effect of estrogen deprivation resulting from use of the aromatase inhibitor exemestane. That study involves American Joint Committee on Cancer/TNM stage I and stage II postmenopausal breast cancer patients who have completed at least 5 years of TAM therapy. Another trial compares the worth of the pure antiestrogen Faslodex (AstraZeneca, Wilmington, DE) with or without chemotherapy versus TAM with or without chemotherapy in premenopausal and postmenopausal, clinically lymph node-negative breast cancer patients.

The NSABP is also interested in studies that evaluate luteinizing hormone-releasing hormone analogues such as Goserlin (AstraZeneca). Another NSABP trial, B-34, compares adjuvant

clodronate therapy with placebo in early-stage breast cancer patients who receive either systemic chemotherapy and/or TAM, or no therapy. The principal aim of that study is to determine whether the second generation bisphosphonate (clodronate) will improve disease-free survival and reduce the incidence of skeletal metastases.

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NOTES

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Ovarian Ablation as Adjuvant Therapy for Breast Cancer

Nancy E. Davidson

Ovarian ablation was the first form of systemic treatment for breast cancer. Its efficacy as a palliative treatment for young women with metastatic breast cancer was initially described by Beatson (1) in 1896. Its use as a form of adjuvant therapy was suggested shortly thereafter, and the first randomized trials of ovarian ablation in the adjuvant setting began in 1948. Although many of these early trials were small and methodologically flawed by modern standards, their combined analysis through the Early Breast Cancer Trialists' Collaborative Group (EBCTG) has unequivocally established that ovarian ablation as a single intervention reduces recurrence and increases survival for women under the age of 50 years (2). Indeed, the magnitude of the benefit is similar to that seen with adjuvant chemotherapy or tamoxifen by indirect comparison (3,4). Thus, the possibility that the benefit conferred by adjuvant chemotherapy is in part because of its ability to induce ovarian failure has been raised.

Adjuvant studies of ovarian ablation during the last 50 years have focused largely on three major questions. The first trials examined the utility of ovarian ablation versus no postoperative therapy. Increasing use of chemotherapy and the recognition that part of its effects could be related to a chemical castration led to randomized comparisons of adjuvant chemotherapy and ovarian ablation with or without tamoxifen. Finally, several recent trials have examined the possibility that ovarian ablation has additional benefit in young women who have received adjuvant chemotherapy. It is surprising that little information is available comparing ovarian ablation and tamoxifen in the adjuvant setting.

FORMS OF OVARIAN ABLATION

Surgical oophorectomy was the original form of ovarian ablation. Its advantage is that it causes an immediate and permanent drop in ovarian steroid production. In the past, the surgical complication rate was relatively high, but current methods of laparoscopic surgery have dramatically reduced postoperative morbidity and mortality. Oophorectomy has the second theoretical advantage of reducing risk of ovarian cancer, which could be potentially useful in women who are genetically predisposed to ovarian cancer.

The advent of therapeutic radiation led to the design of trials of radiation-induced ovarian ablation. Treatment algorithms were variable, ranging from 450 cGy in one fraction to 1000–2000 cGy over five to six fractions. Radiation-induced ovarian ablation is a safe and simple outpatient approach. Its disadvantage is that it may be incomplete or reversible in some women.

During the last 20 years, medical ovarian ablation has emerged as a treatment strategy. A number of analogues of luteinizing hormone-releasing hormone (LHRH) have been studied as treatment for breast cancer. Chronic administration of the LHRH agonists leads to a temporary chemical castration. Ovarian production of estrogen is governed by the pulsatile release of LHRH from the hypothalamus. This leads to pituitary

production of gonadotropins that then act on the ovary to stimulate steroidogenesis. LHRH agonists bind to pituitary gonadotropin receptors more avidly than to native LHRH. This leads to an initial surge in gonadotropin production, but loss of pituitary LHRH receptors, diminished gonadotropin secretion, and cessation of ovarian steroid production then ensue. Two LHRH analogues are available in the United States (goserelin and leuprolide) and can be administered by monthly injection; neither is currently approved by the U.S. Food and Drug Administration for adjuvant therapy of breast cancer. The possible advantages of LHRH analogues are their ease of administration and reversible effects. Side effects are largely those of menopause.

The relative efficacy of surgical, therapeutic radiation, and medical ovarian ablation is not well defined. Indeed, it is generally assumed that these three modalities are equally effective in breast cancer, and the approaches are frequently considered and used interchangeably. Small comparative trials of goserelin with surgery or therapeutic radiation-induced ovarian ablation for treatment of advanced breast cancer have supported this supposition, but it is not known if these results suggesting equivalence in metastatic breast cancer can be extrapolated to the adjuvant setting, where duration of treatment is potentially critical (5).

Cytotoxic chemotherapy represents a fourth form of ovarian ablation because of its capacity to cause temporary or permanent ovarian dysfunction in a substantial number of premenopausal women. The risk of chemotherapy-related amenorrhea is directly related to age at time of treatment and varies with type, dose, and duration of chemotherapy. In general, less than 50% of women under 40 years of age will be rendered postmenopausal by standard adjuvant chemotherapy regimens, whereas the majority of women aged 40 or more years of age will become permanently menopausal. Rates of permanent amenorrhea have been reported for several commonly used adjuvant chemotherapy regimens. They range from about 40% after four cycles of doxorubicin and cyclophosphamide (AC) with or without four cycles of paclitaxel to nearly 70% for six cycles of oral cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) (6–8). Thus, ovarian ablation is potentially an indirect effect of adjuvant chemotherapy.

ROLE OF OVARIAN ABLATION

The largest body of data regarding the use of ovarian ablation for adjuvant therapy is the EBCTG meta-analysis. Results for the 15-year analysis were collected in 1995 and published in 1996 (2). Updated data from the 2000 EBCTG meta-analysis have been presented in a preliminary fashion, but analysis is still incomplete. The 1995 overview summarized results from 12 of

Correspondence to: Nancy E. Davidson, M.D., The Johns Hopkins Comprehensive Cancer Center, 1650 Orleans St., Rm. 409, Baltimore, MD 21231 (e-mail: davidna@jhmi.edu).

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the 13 randomized studies that assessed ovarian ablation by surgery or irradiation. These trials began before 1980. They enrolled 2102 women under age 50 years and 1354 women greater than or equal to 50 years old at randomization. Analysis was focused on the younger women, since no statistically significant impact of ovarian ablation on outcome for the older women (who were presumably mostly postmenopausal) was observed. Five of the trials included routine use of adjuvant chemotherapy, whereas the other trials did not.

Selected results from the 1995 EBCTG overview analysis are presented in Table 1. Ovarian ablation led to a $25\% \pm 7\%$ reduction in the annual odds of recurrence and a $24\% \pm 7\%$ reduction in the annual odds of death for women who underwent ovarian ablation in the absence of chemotherapy. The benefit is seen in women with both lymph node-negative and lymph node-positive disease. There was a trend for greater efficacy of ablation in women with estrogen receptor (ER)-positive tumors, although this parameter was assessed only in four of the 12 trials, all of which included chemotherapy. The benefit was less pronounced for women who were randomly assigned to ovarian ablation in the presence of chemotherapy ($10\% \pm 9\%$ reduction for recurrence and $8\% \pm 10\%$ reduction in mortality). These results likely reflect, in part, the fact that many women became postmenopausal as a consequence of adjuvant chemotherapy. Taken together, these analyses established the clinical benefit of ovarian ablation as an adjuvant approach for young women with breast cancer. It is important that this analysis found no difference in vascular deaths or other non-breast cancer deaths between women allocated to ovarian ablation and those who were not so allocated.

A number of issues complicate interpretation of these results. Most of these trials were small, limiting statistical power. The analysis was based on patient age rather than on actual menopausal status. The hormone receptor status was frequently not available in these older trials. Since it is probable that the benefits of ovarian ablation are most pronounced in premenopausal women with steroid receptor-positive tumors, the true magnitude of benefit might be diluted. Further, these trials did not address other questions of interest, including 1) the relative efficacy of ovarian ablation and chemotherapy, 2) the value of ovarian ablation in conjunction with chemotherapy, 3) the utility of combined endocrine therapy with ovarian ablation and tamoxifen, and 4) the use of temporary ovarian ablation through the application of LHRH agonists. A number of trials conducted during the last 20 years have begun to shed light on these issues.

Table 1. Selected results from the Early Breast Cancer Trialists' Collaborative Group overview analysis of ovarian ablation for women under 50 years of age*

Patients	15-y disease-free survival, %	15-y survival, %
All		
Control	39	46
Ovarian ablation	45 } $P < .001$	52 } $P = .001$
Lymph node-negative—no chemotherapy		
Control	67	71
Ovarian ablation	75 } $P = .01$	77 } $P = .01$
Lymph node-positive—no chemotherapy		
Control	24	29
Ovarian ablation	37 } $P < .001$	42 } $P < .001$

*Data from (2).

COMPARISONS OF OVARIAN ABLATION AND CHEMOTHERAPY

The recognition that the clinical benefits of adjuvant ovarian ablation and chemotherapy were of similar magnitude by indirect comparison for young women led to several trials comparing these two interventions. A key feature of these newer trials is that they generally define eligibility based on menopausal status rather than on age and frequently restrict entry to women with receptor-positive breast cancer, thus targeting the women who might most likely benefit from an endocrine approach. These trials are summarized in Table 2.

Three trials have compared adjuvant ovarian ablation using different modalities with CMF chemotherapy. The Scottish/Imperial Cancer Research Fund trial randomly assigned 332 premenopausal women with lymph node-positive breast cancer to oophorectomy (with or without prednisolone) or to six to eight cycles of intravenous CMF (with or without prednisolone) (9). There was no difference in event-free or overall survival after a maximum follow-up of 12 years. ER assays were available for 270 tumors. Retrospective analyses of these tissues suggested that oophorectomy was associated with improved survival in patients with ER concentrations greater than or equal to 20 fmol/mg protein, whereas 6 months of oral CMF was more beneficial for patients with ER less than 20 fmol/mg protein.

A second trial assessed radiation-induced ovarian ablation or nine cycles of intravenous CMF in 732 women with hormone receptor-positive breast cancer that involved axillary lymph nodes or measured more than 5 cm (10). The 5-year disease-free survival rate was 67% with ovarian ablation and 66% with CMF. Corresponding survival rates were 78% and 82%, respectively. These differences were not statistically significant. Of note is that amenorrhea occurred in 68% of the patients who received CMF.

The largest trial of this design, the Zoladex Early Breast Cancer Research Association (ZEBRA) Trial, compared six cycles of oral CMF with 2 years of monthly doses of goserelin in 1640 premenopausal women with lymph node-positive tumors (11). About 80% of the patients had ER-positive tumors and, with a median follow-up of 6 years, disease-free (DFS) and overall survival were equivalent for the two treatments in these women. In contrast, CMF led to a statistically significantly longer DFS and overall survival than goserelin for women with ER-negative breast cancer. In this study, about two thirds of patients regained menstrual function within a year after completion of 2 years of goserelin, whereas 80% of women treated with CMF remained amenorrheic at 3 years. Subset analysis suggested that patients treated with CMF who became amenorrheic after therapy had a longer DFS than those who did not. Patient-assessed quality of life was better for goserelin than for CMF during the first 6 months of treatment. Bone loss was substantial with both treatments and improved after completion of goserelin but not after completion of CMF. These results suggest that temporary ovarian ablation using goserelin is a reasonable alternative to CMF chemotherapy for women with lymph node-positive ER-positive breast cancer.

None of these trials used tamoxifen. However, the concept of combined endocrine therapy versus CMF has also been tested in two randomized trials. The Italian Breast Cancer Adjuvant Chemotherapy Cooperative Group 02 study compared oral CMF for 6 months with the combination of ovarian ablation and

Table 2. Randomized trials of chemotherapy versus ovarian ablation with or without tamoxifen as adjuvant therapy^a

Study	Patients	No.	Treatments	Results	Reference Nos.
Scottish	Lymph node-positive	332	CMF × 6–8 Oophorectomy	No difference	(9)
Scandinavian	Stage II, receptor-positive	732	CMF × 9 XRT	No difference	(10)
ZEBRA	Lymph node-positive	1640	CMF × 6 Goserelin × 2 y	No difference for ER-positive; CMF better for ER-negative	(11)
GROCTA 02	Lymph node-positive, receptor-positive	235	CMF × 6 Ovarian ablation + tamoxifen × 5 y	No difference	(12)
ABCSG 5	Stage I/II, receptor-positive	1045	CMF × 6 Goserelin × 3 y + tamoxifen × 5 y	Better RFS with endocrine therapy	(13,14)
French	Lymph node-positive, ER-positive	162	FAC × 6 Ovarian ablation + tamoxifen	No difference	(15)
FASG 06	Lymph node-positive, ER-positive	333	FEC × 6 Triptoreline + tamoxifen × 5 y	No difference	(16)

*Abbreviations used: C = cyclophosphamide; A = doxorubicin; F = 5-fluorouracil; M = methotrexate; E = epirubicin; XRT = radiation therapy; ER = estrogen receptor; RFS = recurrence-free survival; ZEBRA = Zoladex Early Breast Cancer Research Association; GROCTA = Italian Breast Cancer Adjuvant Chemo-Hormone Therapy Cooperative Group; ABCSG = Austrian Breast Cancer Study Group; FASG = French Adjuvant Study Group.

5 years of tamoxifen in 244 premenopausal women with axillary lymph node-positive, steroid receptor-positive breast cancer (12). Ovarian ablation could be carried out by surgery, radiation therapy, or 2 years of goserelin. With a median follow-up of 76 months, there was no difference in DFS or overall survival. There was no obvious difference in outcome with any type of ovarian ablation, although the power of this subset analysis is severely limited by the small sample size.

A larger trial of similar design was conducted by the Austrian Breast Cancer Study Group (ABCSG) (13,14). ABCSG 5 compared intravenous CMF for six cycles with goserelin for 3 years and tamoxifen for 5 years in 1045 women with stage I or II steroid receptor-positive breast cancer. At a median follow-up of 42 months, combination endocrine therapy showed a statistically significantly improved recurrence-free survival (RFS) compared with CMF ($P = .02$), but there was no difference in survival. In the CMF group, those women who developed amenorrhea had significantly longer RFS and survival than those who did not. Indeed, the difference favoring combined endocrine therapy in this trial as opposed to the other four CMF trials summarized above may reflect in part a lower likelihood of becoming amenorrheic with six cycles of intravenous CMF compared with six cycles of oral CMF. The addition of tamoxifen to the ovarian ablation but not to the chemotherapy arm may also play an important role.

Taken together, results from these five trials suggest that ovarian ablation with or without tamoxifen can provide clinical benefit similar to that seen with 6 months of oral CMF chemotherapy in women with lymph node-positive, receptor-positive breast cancer. In no case was tamoxifen administered to the women who received chemotherapy, a barrier to extrapolation of these results to current adjuvant therapy practices. In addition, given the apparent superiority of anthracycline-containing regimens compared with CMF in the adjuvant setting, a relevant question is whether similar results would be obtained in a comparison of anthracycline-containing combination chemotherapy and ovarian ablation. This question has not been as well studied.

One small French trial compared adjuvant chemotherapy with 5-fluorouracil, doxorubicin, and cyclophosphamide (FAC) with ovarian ablation (oophorectomy or radiotherapy) plus tamoxifen in 162 premenopausal women with lymph node-positive, receptor-positive breast cancer (15). There was no statistically sig-

nificant difference in DFS or survival in this trial, which stopped early because of poor accrual. A second trial, French Adjuvant Study Group (FASG) 06, compared combined hormonal therapy with 3 years of tamoxifen and triptoreline (another investigational LHRH agonist) with six cycles of 5-fluorouracil, epirubicin, and cyclophosphamide (FEC) in 333 premenopausal women with hormone receptor-positive breast cancer involving one to three axillary lymph nodes. With a median follow-up of 54 months, DFS and overall survival were 92% and 97%, respectively, for endocrine therapy and 81% and 93%, respectively, for FEC. These apparent differences were not statistically significant. FEC induced amenorrhea in 42% of patients, a lower incidence than that seen with six cycles of oral CMF (16). Thus, although the available information is more limited, there does not appear to be a substantial difference in outcome for premenopausal women with receptor-positive, lymph node-positive breast cancer who were randomly assigned either to an anthracycline-containing chemotherapy regimen or to ovarian ablation plus tamoxifen.

ADJUVANT CHEMOTHERAPY AND OVARIAN ABLATION

Adjuvant chemotherapy is a routine part of care for many women with early-stage breast cancer. Thus, a major question is whether the use of ovarian ablation adds to the benefits of adjuvant chemotherapy. This question was addressed in Intergroup (INT) 0101, a trial of chemohormonal therapy in 1503 premenopausal women with lymph node-positive, receptor-positive breast cancer. The trial compared three treatment arms: six cycles of oral CAF, six cycles of CAF followed by 5 years of goserelin (CAFZ), and six cycles of CAF followed by 5 years of goserelin and tamoxifen (CAFZT) (17). The 5-year DFS rates were 67% for CAF, 70% for CAFZ, and 77% for CAFZT, whereas 5-year survival was about 85% for all three arms. Comparison of CAFZT with CAFZ showed a statistically significant DFS advantage with the addition of tamoxifen, whereas comparison of CAFZ with CAF showed no DFS advantage for the addition of goserelin. Preliminary retrospective subset analyses suggest that addition of goserelin may be more beneficial in women younger than 40 years of age at trial entry—those women least likely to become postmenopausal after chemotherapy. Final analysis of the impact of amenorrhea, patient age, and serum hormone levels on clinical outcome is in progress.

The Zoladex in Premenopausal Patients (ZIPP) study also permitted assessment of the effects of ovarian ablation in the context of other adjuvant therapy (18,19). That study combined results from four trial groups that used a common 2 × 2 factorial design to evaluate tamoxifen for 2 years, goserelin for 2 years, tamoxifen and goserelin for 2 years, and no endocrine therapy at all in 2648 premenopausal women with early-stage breast cancer of any steroid receptor type. Forty-two percent of women had lymph node-positive disease, and 56% had ER-positive breast cancer. Elective adjuvant chemotherapy was permitted in selected patients according to predetermined plans at each center and was given to 43% of the participants. At a median follow-up of 4.2 years, there was a statistically significant 23% reduction in first events in women who received goserelin (first events in 20% of patients receiving goserelin and 25% of patients not receiving goserelin, $P = .001$). The benefit was less pronounced in patients who received concurrent adjuvant tamoxifen or chemotherapy. There is no statistically significant effect on survival at this time ($P = .12$).

A third trial, International Breast Cancer Study Group (IBCSG) VIII, is evaluating whether the combination of CMF followed by goserelin can improve outcome compared with either modality alone in the treatment of premenopausal women with lymph node-negative breast cancer. After surgery, patients are randomly assigned to receive either goserelin for 2 years or six cycles of oral CMF or six cycles of CMF followed by 1.5 years of goserelin. Accrual is complete, and the trial remains in blinded follow-up.

Data addressing the value of ovarian ablation after chemotherapy are less robust than those comparing effects of ovarian ablation and chemotherapy. At present, however, they do not provide convincing evidence that both modalities should be routinely employed.

OVARIAN ABLATION AND TAMOXIFEN

For many years, tamoxifen was not used as adjuvant therapy for premenopausal women because of the belief that it was ineffective in these younger women. The 1995 EBCTG overview demonstrated that this belief was erroneous, because premenopausal women with ER-positive breast cancer derived a substantial benefit from tamoxifen for 5 years. Unfortunately, virtually all adjuvant trials of ovarian ablation were begun before this fact became widely known. Thus, there is essentially no information from randomized trials about the relative effects of tamoxifen, ovarian ablation, or the combination in premenopausal women with early-stage breast cancer. Since tamoxifen has become a mainstay of the management of premenopausal women with receptor-positive breast cancer, this is a critical and unfortunate deficit. As noted above, INT 0101 showed that the addition of tamoxifen to CAFZ improved outcome in premenopausal women with lymph node-positive, receptor-positive breast cancer. Unfortunately, an arm comparing CAF followed by 5 years of tamoxifen is not available to permit direct assessment of CAFZ, CAFZT, and CAF followed by tamoxifen.

Two trials are investigating ovarian ablation in the absence of chemotherapy. An Intergroup trial enrolled about 350 premenopausal women with lymph node-negative, receptor-positive breast cancer measuring less than 3 cm. Patients were randomly assigned to receive tamoxifen for 5 years or tamoxifen for 5 years plus any form of ovarian ablation (surgery, radiation therapy, or LHRH agonist for 5 years). The trial was closed

prematurely because of poor accrual in an era of increasing chemotherapy use, and it continues in blinded follow-up. A second trial of combined endocrine therapy has completed accrual in Vietnam. Over 700 premenopausal women with early-stage breast cancer clinically have been randomly assigned to oophorectomy and 5 years of tamoxifen either at the time of mastectomy or at the time of relapse. Preliminary results of this trial suggest that adjuvant oophorectomy and tamoxifen led to a statistically significant improvement in 5-year DFS and overall survival compared with initial observation. Only women with steroid receptor-positive tumors benefited from the adjuvant hormone therapy (20).

CONCLUSIONS AND FUTURE DIRECTIONS

The results from the EBCTG meta-analysis suggest that ovarian ablation represents an effective form of adjuvant systemic therapy for premenopausal women. A number of randomized trials have shown that ovarian ablation with or without tamoxifen and standard chemotherapy regimens like CMF have similar benefits for premenopausal women with early-stage receptor-positive breast cancer. Thus, both the 2001 St. Gallen expert panel (21) and the 2000 National Institutes of Health Consensus Development Conference on Adjuvant Therapy for Breast Cancer (22) have suggested that ovarian ablation is a reasonable adjuvant treatment option for premenopausal women with receptor-positive breast cancer. It appears unlikely, however, that ovarian ablation has any benefit for women with receptor-negative breast cancer.

A number of questions remain to be answered. These include the following: 1) the importance of amenorrhea as a determinant for premenopausal women with early-stage breast cancer; 2) the optimal duration of ovarian ablation if an LHRH analogue is employed; 3) the value of ovarian ablation after chemotherapy, particularly for women who remain premenopausal after adjuvant chemotherapy; 4) the utility of combined hormone therapy such as ovarian ablation with tamoxifen or aromatase inhibitors; and 5) careful delineation of the long-term side effects of this endocrine approach.

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Progress in Systemic Chemotherapy of Primary Breast Cancer: an Overview

Gabriel N. Hortobagyi

Substantial progress has been made in the multidisciplinary management of primary breast cancer during the last 30 years. Adjuvant chemotherapy has been shown to significantly reduce the annual risk of cancer recurrence and mortality, and these effects persist even 15 years after diagnosis. Combination chemotherapy is superior to single-agent therapy and anthracycline-containing regimens. Those that combine an anthracycline with 5-fluorouracil and cyclophosphamide are more effective than regimens without an anthracycline. Six cycles of a single regimen appear to provide optimal benefit. Dose reductions below the standard range are associated with inferior results. Dose increases that require growth factor or hematopoietic stem cell support are under investigation; at this time, the existing results provide no compelling reason to use this strategy outside a clinical trial. Regimens using fixed crossover designs with two non-cross-resistant regimens are being evaluated. The addition of a taxane to anthracycline-containing regimens is currently under intense scrutiny, and preliminary analysis of the first three clinical trials has shown encouraging, albeit not compelling, results. For patients with estrogen receptor-positive breast cancer, the sequential administration of chemotherapy and 5 years of tamoxifen therapy provides additive benefits. No compelling evidence exists to combine ovarian ablation with chemotherapy. Most side effects and toxic effects are self-limited, although premature menopause requires monitoring and preventive interventions to preserve bone mineral density. The small risk of acute leukemia is of concern, and additional research to develop safer regimens is clearly indicated. The overall effect of optimal local/regional treatment combined with an anthracycline-containing adjuvant chemotherapy and a taxane (and, for patients with estrogen receptor-positive tumors, 5 years of tamoxifen therapy) is a greater than 50% reduction in annual risks of recurrence of and death from breast cancer. For most patients at intermediate or high risk of cancer recurrence, the benefits of adjuvant chemotherapy exceed by far its unwanted effects. [*J Natl Cancer Inst Monogr* 2001;30:72-9]

During the last three decades of the twentieth century, substantial progress has been made in our understanding of the biology and natural history of primary breast cancer. Conceptual progress has led to improvements in the diagnosis, prevention, and treatment of breast cancer. The major tool used to validate improvements in treatment is the randomized clinical trial. Tens of thousands of women and thousands of clinical investigators have contributed to trials that have defined what should represent today's standard of care. The success of screening mammography has been reviewed elsewhere (1,2), and the development of adjuvant hormonal therapy is covered elsewhere in this Monograph (3,4). In this overview, we will summarize the evo-

lution and current status of adjuvant chemotherapy for primary breast cancer.

BACKGROUND

A century of treatment with various forms of total mastectomy and axillary dissection (i.e., Halsted radical, Patey modified radical, extended radical, extended simple, and total mastectomy with axillary dissection) demonstrated that early invasive breast cancer without distant metastases is curable in some patients. However, many patients developed recurrent or metastatic breast cancer despite local treatment with curative intent. Conceptual constructs and experimental data led to the hypothesis that most primary breast cancers are (or become) systemic in nature by the time the diagnosis is made; consequently, regional therapies cannot provide a realistic probability of cure once micrometastatic deposits have been established. The concept of "adjuvant" chemotherapy was first introduced in the 1950s (5,6) to combine optimal local/regional and systemic treatments and, thus, to maximize the probability of cure. The first test of this strategy was the administration of thiopeta or 5-fluorouracil to patients with operable breast cancer during radical mastectomy and each of the 2 following days. Patients receiving thiopeta or 5-fluorouracil were compared with those receiving placebo (5). A transient delay in cancer recurrence was documented in the premenopausal group receiving chemotherapy. Subsequent clinical trials (7-9) established clearly that adjuvant chemotherapy reduced statistically significantly the annual odds of cancer recurrence and death. The Early Breast Cancer Trialists' Collaborative Group (10-13) performed overviews of all randomized clinical trials testing adjuvant chemotherapy and confirmed and extended the observations from individual clinical trials. We will review the existing data in the context of specific and clinically relevant questions.

WHAT IS THE BENEFIT OBTAINED WITH ADJUVANT CHEMOTHERAPY?

The evidence is overwhelming that adjuvant chemotherapy produces a highly statistically significant reduction in the annual odds of cancer recurrence and death (10-13). This significant reduction is observed in all subgroups in which the hypothesis has been adequately tested. Thus, the relative reduction in the odds of cancer recurrence and death is similar in patients with lymph node-negative and lymph node-positive breast cancer. The absolute clinical benefit depends, however, on the initial risk of cancer recurrence and death (Table 1).

Correspondence to: Gabriel N. Hortobagyi, M.D., F.A.C.P., Department of Breast Medical Oncology, Box 424, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030-4009 (e-mail: ghortoba@mdanderson.org).

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Table 1. Relative and absolute reductions in annual odds of recurrence after adjuvant chemotherapy

Disease-free survival after	1 y	2 y	3 y	4 y	5 y
<i>A) Benefit over a 5-y period, assuming a relative reduction in odds of recurrence of 35% and a baseline risk of recurrence of 10%/y</i>					
Surgery	90%	81%	72.9%	65.61%	59.05%
Relative reduction with chemotherapy	35%	35%	35%	35%	35%
Surgery + chemotherapy	93.5%	87.42%	81.74%	76.43%	71.46%
Absolute reduction	3.5%	6.42%	8.84%	10.82%	12.41%
<i>B) Benefit over a 5-y period, assuming a relative reduction in odds of recurrence of 35% and a baseline risk of recurrence of 5%/y</i>					
Surgery	95%	90.25%	85.74%	81.45%	77.38%
Relative reduction with chemotherapy	35%	35%	35%	35%	35%
Surgery + chemotherapy	96.75%	93.61%	90.57%	87.63%	84.78%
Absolute reduction	1.75%	3.36%	4.83%	6.18%	7.40%

IS THE BENEFIT FROM ADJUVANT CHEMOTHERAPY MODIFIED BY PATIENT OR TUMOR CHARACTERISTICS?

Chemotherapy appears to be more effective for women younger than age 50 years than for women older than age 50 years. Although this is the message clearly given by an overview of the world's randomized trials (13), it is not universally confirmed by individual large randomized studies (14,15). Concerns persist about the adequacy of doses delivered to postmenopausal women (as compared with premenopausal women). In many of the earlier studies of adjuvant chemotherapy doses lower than the standard have been associated with inferior results (15–17). Conversely, there are biologic bases for a potentially greater benefit from adjuvant chemotherapy in premenopausal women. Most chemotherapy regimens used in the adjuvant setting contain an alkylating agent. These drugs produce permanent amenorrhea (i.e., premature menopause) in more than two thirds of premenopausal patients. Therefore, chemotherapy has both a cytotoxic and an endocrine effect in premenopausal patients, whereas the endocrine effect would be absent in postmenopausal women (18,19). The effect of adjuvant chemotherapy in women older than age 70 years has not been adequately tested. An important area for research is the state-of-the-art chemotherapy regimens used to treat women older than age 70 years—a thorough evaluation of these treatment plans and their effects is needed.

Another factor that modifies the effect of adjuvant chemotherapy is the estrogen receptor status of the tumor. The reduction in odds of cancer recurrence or death is 30%–40% greater for estrogen receptor-negative primary tumors than for estrogen receptor-rich tumors (13,20). These differences are observed in both younger and older patient groups.

Preliminary data based on retrospective analyses of clinical trials suggest that anthracycline-containing chemotherapy regimens might be particularly effective in treating HER-2-overexpressing tumors. Additional information about this potential interaction will be needed, preferably from prospective trials.

Combination chemotherapy is significantly more effective than single-agent chemotherapy, both in individual clinical trials and in the world overview (13,20–22). Whether the most effective drugs should be combined simultaneously or in sequence is currently under evaluation. Until those studies are completed, the standard of care is to use simultaneous combinations, as is detailed below.

WHAT IS THE OPTIMAL DURATION OF ADJUVANT CHEMOTHERAPY?

The first studies of adjuvant chemotherapy (5,23–25) used a short course of perioperative chemotherapy only. The effects of these interventions were marginal. The demonstration of substantial and reproducible benefit from prolonged combination chemotherapy as well as the greater benefit derived from longer therapy established this latter strategy as the standard of care (8,9). Several randomized clinical trials (13,26–31) addressed this specific issue. The results of these trials can be summarized as follows: The use of a single-combination chemotherapy regimen for longer than 6 months is not more beneficial than 6 months of treatment (13,27–29). Regimens that use chemotherapy for less than 3–4 months appear to be inferior in efficacy (30,31). Therefore, with regimens currently in use (e.g., cyclophosphamide/methotrexate/5-fluorouracil [CMF], cyclophosphamide/doxorubicin/5-fluorouracil [CAF or FAC], or similar epirubicin-containing combinations [CEF or FEC]), six cycles of therapy administered over a 4- to 6-month period appear to be an optimal regimen. In fact, the adequacy of four cycles of doxorubicin/cyclophosphamide should be questioned. Newer regimens that use two different non-cross-resistant regimens may require eight cycles over a 6-month period or a longer period (32–35).

WHAT IS THE OPTIMAL TIMING OF THE INITIATION OF ADJUVANT CHEMOTHERAPY?

Preclinical experiments suggest that the earlier the initiation of chemotherapy in relation to the injection of tumor cells or the resection of the primary lesion, the higher the cure rate (36–38). Clinical trials have not been designed to determine the efficacy of delayed adjuvant chemotherapy. Therefore, virtually all of the data that we have relevant to this question are derived from trials in which eligibility required starting adjuvant chemotherapy within 60 days of the primary surgical procedure. Within this narrow window, there is no evidence that chemotherapy initiated earlier is more beneficial than chemotherapy initiated later. However, a few retrospective analyses (39,40) have suggested that, for high-risk patients, delaying initiation of chemotherapy might be detrimental. Furthermore, a prospective randomized trial comparing postoperative chemotherapy followed by radiotherapy with the reverse sequence of treatment indicated that delaying chemotherapy until the completion of radiotherapy was associated with increased rates of distant metastases and death

(41). On the basis of this limited information, postoperative adjuvant chemotherapy should begin as soon as possible after the surgical procedure and should precede radiotherapy, especially in patients at high risk of recurrence or metastases.

A question regarding the timing of systemic therapy is whether chemotherapy should start even before definitive surgery. Preoperative chemotherapy has several potential advantages, including reduction in tumor volume, facilitation of breast-conserving therapies, and the opportunity it provides to evaluate chemotherapy sensitivity *in vivo* (42,43). Several randomized clinical trials (44–47) have compared the administration of chemotherapy before or after definitive surgical procedures. The largest of these was the National Surgical Adjuvant Breast and Bowel Project (NSABP) protocol B-18 (44,45). All trials reported to date have shown that preoperative chemotherapy produced results that were virtually identical to those obtained with the postoperative administration of the same regimen. In two smaller trials (46,47), a small advantage was associated with preoperative therapy. Although the optimal utilization of preoperative adjuvant chemotherapy is still under investigation, this appears to be a safe and effective alternative to surgery followed by chemotherapy, especially for primary tumors that would require cytotoxic treatment anyway.

WHAT IS THE ROLE OF ANTHRACYCLINES IN THE ADJUVANT THERAPY OF BREAST CANCER?

Until recently, anthracyclines were considered to be the most effective agents for the treatment of metastatic breast cancer. On the basis of randomized trials (13,48,49), it is evident that anthracycline-containing regimens produce higher response rates and longer response durations and survival than regimens that lack an anthracycline. For this reason, anthracyclines were introduced into adjuvant chemotherapy regimens (9,50–57). The adoption of anthracycline-containing adjuvant regimens has been slow, however, because of concerns about cardiotoxicity (58). Although long-term cardiotoxic effects have been described in the pediatric literature, the risk of late cardiotoxic effects remains very low in the adult population, with cumulative doses of doxorubicin or epirubicin limited to well below the cardiotoxic threshold (56,57,59–62). In the metastatic setting, the risk of cardiac toxicity can be reduced by limiting the cumulative dose of the anthracycline (58), by using 48- to 96-hour continuous infusion schedules (63), or by using a cardioprotector (dexrazoxane) (64,65) or an anthracycline with lower cardiotoxic potential [such as epirubicin (66), liposomal doxorubicin (67), or stealth liposomal doxorubicin (68)]. In addition to long-term safety data from a few trials with follow-up periods exceeding 10 years (69,70), there are now more than 15 reported prospective randomized trials of adjuvant chemotherapy regimens that include an anthracycline (52,54–56,71–79). Several individual trials (52,55,57) and the world overview (12,13,20) of all randomized trials have demonstrated that anthracycline-containing chemotherapy regimens are associated with higher disease-free and overall survival rates than regimens that do not include anthracyclines. The annual reduction in odds of recurrence was 12% [standard deviation (SD), 4%]; the reduction in odds of death was 11% [SD, 5% (13)]. However, the clinical trials on which this calculation was based included a variety of anthracycline-containing chemotherapy regimens, some of them considered to be, in retrospect, suboptimal. The first two NSABP protocols that incorporated doxorubicin utilized this drug at 30

mg/m² every 28 days (52). This dose (or dose intensity) is known to be less effective than more commonly used dose regimens (17). Furthermore, in several trials (54,75,77,78), the two-drug (doxorubicin/cyclophosphamide [AC] or epirubicin/cyclophosphamide [EC]) combination was used as the “investigational” arm; this regimen was shown in a large trial (54) to be equivalent to but not better than the CMF combination (54,75,77–79). Neither AC nor EC has ever been directly compared with the more standard three-drug regimens (FAC/CAF or FEC/CEF) that have been shown to produce superior disease-free or overall survival rates when compared with CMF (Table 2). These indirect comparisons between AC and FAC (or the epirubicin equivalents) invite the question of whether AC is equivalent to or inferior to FAC. In the absence of direct comparisons between AC and FAC, prudence dictates that FAC (or CAF, FEC, or CEF) rather than AC be considered the standard anthracycline-based regimen. Whether the apparent superiority of FAC/CAF over AC results from the use of a third drug, 5-fluorouracil, or the longer duration of therapy (usually six to eight cycles of FAC/FEC versus four cycles of AC/EC) is unknown. The determination of the optimal duration of therapy and the optimal number of drugs in anthracycline-containing regimens is an important research question as the choice of the “standard arm” of subsequent trials depends on this answer. Were the results in the taxane arm of Cancer and Leukemia Group B (CALGB) 9344 (32) better than the results for those who received four cycles of AC because a longer duration of therapy was used or because paclitaxel was substituted for 5-fluorouracil?

The recent update of the Scandinavian trial comparing a tailored FEC regimen with four cycles of FEC followed by one cycle of high-dose chemotherapy indicated the superiority of the tailored FEC regimen (80). Should this regimen be the next standard of care and, therefore, the gold standard in future clinical trials?

Additional important research questions remain to be answered concerning the role of other anthracyclines [liposomal anthracyclines (67,68,81) or anthrapyrazoles (82)] in the adjuvant chemotherapy setting.

WHAT IS THE ROLE OF TAXANES IN ADJUVANT CHEMOTHERAPY?

The two commercially available taxanes, paclitaxel and docetaxel, are considered the most effective agents against metastatic breast cancer (83–86). Their efficacy matches, and in some cases exceeds, that of the anthracyclines. Both taxanes have been shown to increase response rates and to prolong response duration and overall survival in some randomized trials (87–91). Therefore, the introduction of taxanes into adjuvant chemotherapy regimens was a logical step. To date, the preliminary results of three randomized trials that evaluated the inclusion of a taxane into adjuvant therapy have been presented (Mamounas EP: Results of National Surgical Adjuvant Breast and Bowel Project protocol B-28, presented at the National Institutes of Health Consensus Development Conference on Adjuvant Therapy for Breast Cancer, Oct. 31–Nov. 3, 2001, Bethesda, MD) (32,92). In essence, the addition of four cycles of paclitaxel to four cycles of AC significantly reduced the annual odds of cancer recurrence and death (32). On the basis of these results, the U.S. Food and Drug Administration approved the use of paclitaxel in the adjuvant chemotherapy setting. The preliminary re-

Table 2. Randomized trials comparing anthracycline-containing with non-anthracycline-containing combination chemotherapy regimens as adjuvant therapy for operable breast cancer*

Author (study)	No. of patients: A/non-A	Regimens†	Year	Lymph node-positive	Median follow-up, mo	Disease-free survival rate		Overall survival rate		Notes
						A	Non-A	A	Non-A	
FAC/CAF/FEC/PAF‡ regimens										
Hutchins (55)	1340/1351	CAF/CMF	1998	None	84	85	82‡	NA	NA	
Mouridsen (76)	1195	CEF/CMF	1999	None	61	NA	NA	93	83	Premenopausal
Fisher (B12) (52)	548/558	PAFT/PFT	1989	All	64	64	63	77	78‡	Postmenopausal or ER positive
Fisher (B11) (52)	347/360	PAF/PF	1989	All	51	51	44‡	65	59‡	Premenopausal or ER negative
Levine (MA5) (73)	351/359	CEF/CMF	1998	All	60	63	53‡	77	70‡	Premenopausal
Carpenter (71)	260/268	CAF/CMF	1991	All	42	NA	NA	71	70	Premenopausal
Coombes (72)	380/379	FEC/CMF	1996	All	54	NA§	NA§	NA§	NA§	Premenopausal
AC or EC vs. CMF										
Fisher (B23) (78)	1003/1005	AC/CMF	2000	None	67	82	82	88	90	
Fisher (B15) (54)	781/776	AC/CMF	1990	All	26	62	63	83	82	
Di Leo (75)	562/255	EC/CMF	1999	All	50	64–74	71	78–86	85	
Gallioioni (77)	103/104	EC/CMF	1997	All	36	72	63	91	89	Premenopausal

*A = anthracycline-containing regimen; ER = estrogen receptor.

†FAC = 5-fluorouracil, doxorubicin (i.e., Adriamycin), and cyclophosphamide; CAF = cyclophosphamide, doxorubicin, and 5-fluorouracil; FEC = 5-fluorouracil, epirubicin, and cyclophosphamide; PAF = L-phenylalanine mustard, doxorubicin, and 5-fluorouracil; T = tamoxifen; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; NA = not available.

‡Statistically significant difference (two-sided $P < .05$).

§Separate sets of numbers given for different schedules of FEC and CMF. FEC1 (the dose schedule used in the first half of the study by Coombes) and CMF1 (the dose schedule used in the first half of the study by Coombes) appear equivalent. FEC2 (the dose schedule used in the second half of the study by Coombes) appears to be superior to CMF2 (the dose schedule used in the second half of the study by Coombes) in disease-free survival rate and overall survival rate.

sults of The University of Texas M. D. Anderson Cancer Center trial (92) and the NSABP protocol B-28(REF) show, as yet, no statistically significant differences; however, both trials show results that are statistically consistent with the results of CALGB 9344. Additional follow-up of these three trials, as well as of the many others in the recruitment phase, will provide the definitive answer about the role of these highly effective drugs in managing primary breast cancer.

IS THE DOSE OF CHEMOTHERAPY AN IMPORTANT DETERMINANT OF OUTCOME AFTER ADJUVANT CHEMOTHERAPY?

The answer to this question is, certainly, yes; however, it is a highly qualified yes. Extensive data from preclinical experiments show a direct correlation between dose and extent of cytotoxicity (93); however, there are only a few well-designed clinical trials to address the issue of dose. In both the metastatic and the adjuvant setting, there are data to show that variations in dose or dose intensity are important. It is unclear from published studies whether dose, dose intensity, or dose density is the most influential factor. However, the data can be interpreted to show that reductions in dose below the optimal dose possible without hematopoietic cell or growth factor support result in decreased benefit (17). These studies are consistent with the existence of a dose threshold below which the treatment loses its effectiveness. Therefore, recommended doses for commonly used regimens should not be altered unless the patient's safety requires it. Retrospective subset analyses suggest that dose reductions would have an adverse effect for HER-2-positive but not for HER-2-negative tumors. Above the standard dose range, existing data are inconclusive. Five prospective randomized trials (80,94–97) have been reported to date that compare a "standard dose" regimen with a high-dose regimen. All five trials required hemato-

poietic stem cell and growth factor support. Preliminary analyses in two of the trials (96,97) suggested a decrease in the number of recurrences in the group receiving the high-dose therapy, whereas there was no detectable difference in the other three studies. Several additional randomized trials addressing this important question are still in the patient-recruitment phase (98). Mature results of all these completed and ongoing trials will determine whether high-dose chemotherapy has a role in the management of patients with high-risk primary breast cancer. At the moment, there is no indication that high-dose chemotherapy should be used outside a well-designed clinical trial.

DOES THE COMBINATION OF ADJUVANT CHEMOTHERAPY AND HORMONE THERAPY PRODUCE SUPERIOR RESULTS TO CHEMOTHERAPY ALONE OR TO HORMONE THERAPY ALONE?

This question is restricted to patients with hormone receptor-positive breast cancer, since hormone therapy produces no benefit in women with hormone receptor-negative tumors (3). Several randomized trials have addressed this issue in women with hormone receptor-positive (or unknown) breast cancer. The last two world overviews have also analyzed the pooled data from these trials (13). The addition of tamoxifen therapy to adjuvant chemotherapy statistically significantly increases the disease-free and overall survival rates of patients with hormone receptor-positive tumors. This effect is observed regardless of age or lymph node status and appears to be of the same magnitude in both younger women and older women. Conversely, the addition of chemotherapy to tamoxifen therapy statistically significantly increases the results of treatment compared with tamoxifen therapy alone. Therefore, for women with hormone receptor-positive breast cancer, the treatment of choice is the combination of chemotherapy and 5 years of tamoxifen therapy. Most on-

oncologists prefer to administer all chemotherapy first and then start tamoxifen therapy, so as to avoid the increased risk of thromboembolic complications associated with the simultaneous administration of both modes of treatment (99,100).

The combination of chemotherapy with ovarian ablation is currently under intense scrutiny in several randomized trials (101). This topic is reviewed in detail elsewhere in this Monograph (4). At this time, there is no compelling evidence to support the combination of any form of ovarian ablation with chemotherapy. Instead, tamoxifen therapy combined with chemotherapy appears to be the treatment of choice. No information is available about the role of aromatase inhibitors or other endocrine interventions in the adjuvant chemotherapy setting, although the former are currently under investigation in clinical trials.

WHAT IS THE OVERALL BENEFIT OF ADJUVANT SYSTEMIC THERAPY?

Many physicians and patients assume that the results of individual trials comparing two closely related treatments or similar analyses from the world overview (12) represent the best that adjuvant systemic therapy has to offer. In reality, progress is stepwise and incremental. Thus, it is important to combine the effects of all incremental steps to determine the overall impact of combined-modality therapy for primary breast cancer. We do not know the overall impact of optimal surgical resection on disease-free and overall survival, since this has never been (and perhaps will never be) the subject of randomized trials. However, we have ample evidence that the addition of postoperative radiation therapy to surgery statistically significantly reduces the risk of recurrence and mortality from breast cancer (102). Furthermore, we have compelling evidence that first-generation chemotherapy regimens (CMF and related combinations) reduce annual odds of cancer recurrence by 23% (SD, 2.1) and odds of death by 15% (SD, 2.4) (13). The addition of an anthracycline further reduces residual risk by 12% and 11%, respectively. These latter figures probably represent an underestimate, based on the caveats expressed in the anthracycline section of this article. On the basis of the results of CALGB 9344, it is probable that adding a taxane to an anthracycline-based regimen will produce an additional reduction in risk of cancer recurrence and mortality. Finally, there is compelling evidence that the combination of 5 years of tamoxifen therapy with chemotherapy results in statistically significant improvements to outcome (3). Therefore, if one uses optimal surgery, radiotherapy, state-of-the-art chemotherapy, and, for hormone receptor-positive tumors, tamoxifen therapy, the overall reduction in risk of cancer recurrence will probably exceed 50%, and the reduction in mortality should also approach that figure. Although individual patients might want to look at the contribution of each component of their treatment, it is helpful to point out the overall benefits obtained with the entire treatment strategy relevant to that patient and her tumor.

WHAT ARE THE SIDE EFFECTS AND THE SHORT-TERM AND LONG-TERM TOXIC EFFECTS OF ADJUVANT SYSTEMIC CHEMOTHERAPY?

This is a critical component of the therapeutic ratio and is reviewed in detail by Dr. Winer's article (103). Most chemotherapy regimens in use today have the potential to produce

nausea, vomiting, mucositis, diarrhea, and alopecia in different degrees (60). All of these effects are self-limited, and we have effective tools to modify or prevent their occurrence. Myelosuppression is common, but neutropenic febrile episodes are uncommon and frank infections are rare. Deaths from toxicity should be exceptional when average-risk patients receive therapy from expert hands. Premature menopause is the most common long-term toxicity for premenopausal women (60, 104). Emerging data suggest that this might be a desirable effect of chemotherapy, which is associated with improved outcomes. Menopausal symptoms can be successfully controlled with medical interventions in a high proportion of patients, while the long-term effects of premature menopause on cardiovascular risk and the early onset of osteoporosis can be successfully managed with statins and bisphosphonates or selective estrogen receptor modulators, respectively.

The development of myelodysplastic syndromes or acute leukemia is a known (albeit rare) complication of chemotherapy with alkylating agents and topoisomerase II inhibitors (105–107). The cumulative incidence of these disorders is around 1% for commonly employed regimens. These events are, however, life threatening, so the development of even safer chemotherapy regimens or the identification of patients at greater than average risk for these complications should be a high priority of research in the near future.

SELECTION OF OPTIMAL ADJUVANT SYSTEMIC THERAPY

Because of our improved ability to reduce the risk of recurrence and, especially, of death, most patients with primary invasive breast cancer should receive multidisciplinary treatments. Thus, it is accepted practice that all patients with a calculated risk of recurrence exceeding 10% over a 10-year period should be advised to receive adjuvant systemic therapy. Most otherwise healthy patients with a calculated risk between 5% and 10% should have their available treatment options discussed with them; many will choose to receive hormone therapy, chemotherapy, or a combination of both. Once a decision has been made about the need for or desirability of adjuvant systemic therapies, the following guidelines should be considered:

- 1) All patients with hormone receptor-positive tumors should receive tamoxifen therapy for 5 years. Patients with hormone receptor-negative tumors should not be offered adjuvant hormone therapies.
- 2) The treatment of choice for patients with hormone receptor-negative tumors is combination chemotherapy, preferably with an anthracycline-containing regimen. The three-drug regimens (FAC, CAF, FEC, or CEF) are preferred to AC or EC. Six cycles of therapy with a single regimen is probably optimal.
- 3) The addition of taxanes should be considered for high-risk patients with lymph node-positive breast cancer, especially if the cancer is hormone receptor negative.
- 4) For most patients at intermediate or high risk of recurrence and of death and with hormone receptor-positive tumors, the sequential combination of chemotherapy and tamoxifen therapy for 5 years represents the treatment of choice.
- 5) The use of optimally performed interventions provides maximal benefits. Chemotherapy is no substitute for good surgery, or vice versa.

6) The combined use of optimal surgery, chemotherapy, tamoxifen therapy, and radiotherapy has a major impact on the risk of recurrence and death.

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Is Her2 of Value in Identifying Patients Who Particularly Benefit From Anthracyclines During Adjuvant Therapy? A Qualified Yes.

Peter Marcus Ravdin

Data from several large adjuvant breast cancer chemotherapy trials suggest that anthracycline-based chemotherapies relative to non-anthracycline-based adjuvant therapies are particularly effective in patients whose tumors overexpress Her2. Most trials show some evidence of this effect, but the interaction generally has not been confirmed statistically, perhaps because the trials are underpowered. In addition, there have been a multiplicity of Her2 immunohistochemistry techniques used in these studies, which are clearly not of equivalent utility in detecting this effect. Thus, while there is good evidence that further work in this area will be of value, at this time the results are inconclusive and not ready for clinical application. [J Natl Cancer Inst Monogr 2001;30:80-4]

INTRODUCTION

In this article, I will argue for and review the clinical trial evidence for the value of Her2 overexpression to identify breast cancer patients who particularly benefit from the inclusion of anthracyclines in adjuvant therapy.

DEALING WITH COUNTERARGUMENTS

The problem areas for my argument include 1) questions about the methodologies for measuring Her2 overexpression, 2) the apparent discordance between the possible ability of Her2 to select patients who particularly benefit from anthracyclines in adjuvant therapy and its apparent lack of predictive value for selecting patients likely to benefit from anthracycline-based therapy in metastatic disease, 3) the lack of a clear mechanism by which Her2 overexpression should lead to particular anthracycline sensitivity, and 4) some clinical studies (underpowered) that do not support the predictive value of Her2 in anthracycline-based adjuvant therapy.

The weakest element is that there are a number of techniques for detecting and scoring Her2 overexpression in breast tissue samples. At present, nearly all of the work on the predictive value of Her2 has been done using immunohistochemistry (IHC). Clearly, not all immunohistochemical techniques are equivalent; they differ in primary and secondary antibodies used, antigen retrieval methods used, scoring and interpretation of overexpression, and so forth. This complicates the clinical use of Her2 information, as is beautifully illustrated in some of the studies I will review, in which, for patients from the same clinical study, one immunohistochemical technique seems to be predictive of particular benefit of anthracycline-based adjuvant therapy, whereas another Her2 IHC technique used on the same samples was not predictive.

A second counterargument against the use of Her2 for predicting adjuvant benefit of anthracyclines is that Her2 is not a

recognized predictor of treatment benefit of anthracyclines in metastatic disease. This is an important point, with most studies not finding Her2 to be a predictor of response to anthracycline-based therapy in metastatic disease. It should be noted that most of the studies in which there was no correlation were quite small [fewer than 100 patients; e.g., studies with only 54 (1), 23 (2), 103 (3), and 60 cases (4)] and, thus, had a very modest statistical power to find correlation between Her2 overexpression and response. To counter this argument, it must be argued that the tumor cells are in a different state when they are part of a macroscopic tumor mass compared with when they are part of a micrometastatic tumor burden. The observation of response in metastatic disease depends on the effects on cell kill as balanced against tumor growth. By promoting rapid regrowth, a marker like Her2 can obscure the benefit conferred by an agent in metastatic disease. Perhaps this effect of rapid regrowth is less prominent in the adjuvant setting. There are some clinical lines of evidence that such a differential effect may occur. For example, in the overview analysis (5), there are repeated trends toward substantially more benefit from adjuvant chemotherapy in estrogen receptor-negative versus estrogen receptor-positive patients, although estrogen receptor status is not an accepted predictor of response to chemotherapy in metastatic disease.

Another argument against Her2 as a marker of anthracycline responsiveness in adjuvant therapy is that why overexpression of Her2 would be associated with anthracycline sensitivity is not yet well understood. There are a number of plausible explanations, but none as yet has been demonstrated convincingly. These explanations range from possible association of Her2 overexpression with topoisomerase II (the target enzyme for anthracyclines) expression (6,7) to a number of other ideas on how Her2 overexpression may activate other pathways that might render the breast cancer cells more sensitive to chemotherapy.

One additional important counterargument that can be used to deflate "negative studies" in which Her2 is not found to be a statistically significant predictor is that nearly all of the studies of the predictive significance of Her2 are dramatically underpowered to find the kind of effects that they are trying to detect. As a general rule of thumb, in comparison with a two-arm trial comparing two treatment regimens, a study looking for interactions between a marker and treatment will need four times as many patients. Statistical power is further weakened by the fact that Her2 overexpression is found in only 20%-30% of breast tumors. Estimates can be made of how many patients are needed to detect statistically significant interactions, and these estimates suggest that more than 1000 patients are needed. Thus, it might

Correspondence to: Peter Ravdin, M.D., Ph.D., Division of Oncology, The University of Texas Health Sciences Center, San Antonio, 7703 Floyd Curl Dr., Rm. 5.214S, San Antonio, TX 78229 (e-mail: pmravdin@aol.com).

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be expected that, even with a real interaction between Her2 and anthracyclines, many studies would not be able to demonstrate this interaction with statistical confidence. Only one study of the interaction between Her2 and anthracycline-based adjuvant therapy has included more than 1000 patients.

CLINICAL TRIAL EVIDENCE

As reviewed below, a picture emerges in which most studies show that subsets of patients whose tumors overexpress Her2 derive particular benefit from anthracyclines. These studies will be reviewed as four different sets of studies: the initial hypothesis generation studies, the National Surgical Adjuvant Breast and Bowel Project (NSABP) trials, two major Cooperative Group studies, and other smaller studies.

First to be reviewed is the Cancer and Leukemia Group B (CALGB) trial that launched a special interest in this area. The second set of trials contains the biologic correlative studies on NSABP B-11 and B-15. These trials are among the largest trials and used exactly the same methodology; thus, to some extent, they cross-validate each other. The third tier of trials contains large trials with more than 500 patients: the Southwest Oncology Group (SWOG)/Intergroup study and a European Consortium study. These two studies used methodologies that, to some extent, were similar to those of the CALGB and NSABP. In addition, by using more than one methodology for Her2 IHC, these two studies attempted to evaluate how generalizable these methodologies are. Finally, in a fourth tier, a review will be presented for the four trials, which supply some relatively weak data from studies of fewer than 400 patients, and with other important nonidealities. Tables 1–3 summarize some of the information from the larger prospective studies.

Initial (Hypothesis-Generating) Results of the CALGB

The first major block of clinical evidence that Her2 might identify a subset of patients particularly likely to benefit from anthracycline-based chemotherapy came from work on specimens from CALGB 8541 (8). In this study, patients with lymph node-positive breast cancer were randomly assigned to receive one of three dose levels of cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF). An initial cohort of 397 cases (of 1572 from the trial) was analyzed. A polyclonal serum was used as the primary antibody for the IHC, and Her2 expression was scored as a continuous variable. In this first cohort of patients, it was noted that the highest dose CAF was of particular value for patients with Her2-overexpressing tumors.

A second cohort of patients was then analyzed. This subset included mostly patients who were entered in the latter half of the patient accrual when estrogen receptor-positive patients were given tamoxifen. In addition, the Her2 IHC analysis was performed differently in the second cohort in this study than it was in the first cohort. In the second cohort, Her2 expression was determined using the monoclonal CB11 rather than the polyclonal antisera. The initial analysis of the second cohort of patients (without inclusion of the first cohort) did not confirm the interaction between Her2 and anthracycline dose seen in the first cohort.

The data were then combined and analyzed as a 992-patient set (9). A special weighting also was made to adjust for some of the imbalances between the cohorts. In this third combined analysis, a statistically significant interaction between anthracycline dose and Her2 expression was noted. This interaction conferred particular benefit for the higher dose of CAF (what we now consider the standard dose) on patients whose tumors overexpress Her2.

The CALGB study has some important weaknesses. Special statistical weightings were made to balance the two cohorts. Different methodologies were used in the two cohorts for the Her2 IHC. The study and its analysis were retrospective and exploratory in character and subject to the biases of such analyses. Thus, this study might be viewed as useful for hypothesis generation; studies more prospective in character are necessary to validate and extend these hypotheses.

NSABP Studies of the Predictive Character of Her2

The strongest of the studies supporting the use of Her2 to select patients who might particularly benefit from anthracycline-based chemotherapy come from the NSABP. The first of their studies was based on NSABP B-11 (10). In this trial, lymph node-positive, steroid hormone receptor-negative patients were randomly assigned to receive either melphalan, doxorubicin, and 5-fluorouracil (PAF) or melphalan and 5-fluorouracil (PF). The NSABP studies have used as antibodies for primary staining a cocktail of a monoclonal MAb-1 and p-Ab1 polyclonal sera. Cases were scored as positive if greater than 1% of the cells were stained. Tumor samples from 638 patients were analyzed. Her2 overexpression was observed in 38% of the cases. The mean time of follow-up was 13.5 years.

The patients with Her2-positive tumors received a higher degree of benefit from PAF than from PF. The improved outcome for Her2-positive patients was reflected in a relative risk

Table 1. Study characteristics of major randomized trials examining the interaction of Her2 expression and added effectiveness of anthracyclines in adjuvant therapy*

Study	Treatment	No.	Median FU, y	Antibody used	Criteria for Her2 positivity	% of cases Her2 positive
B-11	PAF vs. PF	638	13.5	MAb-1 and pAb-1	Any cells with definite membrane staining	37
B-15	AC vs. CMF	1319	12.4	MAb-1 and pAb-1	Any cells with definite membrane staining	29
Euro (1)	EC vs. CMF	481	4.8	4D5 and/or CB-11	Any cells with definite membrane staining	12
Euro (2)	EC vs. CMF	481	4.8	MAb-1 and pAb-1	Any cells with definite membrane staining	18
S8814 (1)	CAF + Tam vs. Tam	746	7.3	MAb-1	Any cells with definite membrane staining	20
S8814 (2)	CAF + Tam vs. Tam	746	7.3	CB11	Combined % staining and staining intensities	23

*PAF = melphalan, doxorubicin, and 5-fluorouracil; PF = melphalan and 5-fluorouracil; AC = doxorubicin and cyclophosphamide; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; EC = epirubicin (60 mg/m²)/cyclophosphamide; CAF = cyclophosphamide, doxorubicin, and 5-fluorouracil; Tam = tamoxifen; FU = follow-up.

Table 2. Disease-free survival rate of major randomized trials examining the interaction of Her2 expression and added effectiveness of anthracyclines in adjuvant therapy*

Study	Treatment	Her2 positive			Her2 negative			Interaction: <i>P</i>
		Relative risk	95% CI	<i>P</i>	Relative risk	95% CI	<i>P</i>	
B-11	PAF vs. PF	0.60	0.42 to 0.82	.002	0.96	0.75 to 1.23	.74	.02
B-15	AC vs. CMF	0.84	0.65 to 1.07	.15	1.02	0.86 to 1.20	.84	.19
Euro (1)	EC vs. CMF	0.33	0.09 to 1.27	.08	1.16	0.71 to 1.90	.56	.10
Euro (2)	EC vs. CMF	1.06	0.45 to 2.52	.90	0.99	0.58 to 1.68	.97	.84
S8814 (1)	CAF + Tam vs. Tam	0.53	0.30 to 0.95	.03	0.85	0.61 to 1.19	.34	.24
S8814 (2)	CAF + Tam vs. Tam	0.70	nr	.28	0.84	0.60 to 1.18	.37	.58

*CI = confidence interval; PAF = melphalan, doxorubicin, and 5-fluorouracil; PF = melphalan and 5-fluorouracil; AC = doxorubicin and cyclophosphamide; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; EC = epirubicin (60 mg/m²)/cyclophosphamide; CAF = cyclophosphamide, doxorubicin, and 5-fluorouracil; Tam = tamoxifen; nr = not reported.

Table 3. Overall survival rate of major randomized trials examining the interaction of Her2 expression and added effectiveness of anthracyclines in adjuvant therapy*

Study	Treatment	Her2 positive			Her2 negative			Interaction: <i>P</i>
		Relative risk	95% CI	<i>P</i>	Relative risk	95% CI	<i>P</i>	
B-11	PAF vs. PF	0.66	0.47 to 0.92	.01	0.90	0.69 to 1.19	.47	.15
B-15	AC vs. CMF	0.82	0.63 to 1.06	.13	1.07	0.88 to 1.30	.84	.11
Euro (1)	EC vs. CMF	nr	nr	nr	nr	nr	nr	nr
Euro (2)	EC vs. CMF	nr	nr	nr	nr	nr	nr	nr
S8814 (1)	CAF + Tam vs. Tam	0.44	0.24 to 0.83	.009	0.96	0.65 to 1.41	.82	.10
S8814 (2)	CAF + Tam vs. Tam	0.79	nr	.41	0.84	0.56 to 1.25	.41	.88

*CI = confidence interval; PAF = melphalan, doxorubicin, and 5-fluorouracil; PF = melphalan and 5-fluorouracil; AC = doxorubicin and cyclophosphamide; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; EC = epirubicin (60 mg/m²)/cyclophosphamide; CAF = cyclophosphamide, doxorubicin, and 5-fluorouracil; Tam = tamoxifen; nr = not reported.

(RR) of relapse, which in the Her2-positive patients was 0.60 (95% confidence interval [CI] = 0.42 to 0.82; *P* = .002), and survival was 0.66 (95% CI = 0.47 to 0.92; *P* = .01). Statistically significant improvements were not seen for patients with Her2-negative tumors with disease-free survival (DFS) showing an RR of relapse of 0.96 (95% CI = 0.75 to 1.23; *P* = ns); and an RR for survival of 0.90 (95% CI = 0.69 to 1.19; *P* = ns). A statistically significant interaction between doxorubicin treatment and erbB-2 overexpression was demonstrated for DFS (*P* = .02), with a trend for such an interaction demonstrated for survival, although this did not reach conventional statistical significance (*P* = .15). The authors of this manuscript concluded that their data supported the hypothesis of a preferential benefit from doxorubicin in patients with Her2-positive breast cancer.

This study was followed by a second NSABP study (11) that used the same techniques (the same antibody mixture for the IHC and the same scoring criteria). This study had a similar result but was less convincing than the B-11 study. The second NSABP study was based on NSABP B-15, in which lymph node-positive patients were randomly assigned to received doxorubicin and cyclophosphamide (AC); cyclophosphamide, methotrexate, and 5-fluorouracil (CMF); or AC followed by CMF. It was hypothesized that AC would be superior to CMF, particularly in the patients whose tumors overexpress Her2. Immunohistochemical Her2 determinations were performed on 1355 cases treated from the AC or CMF arms of the study. Twenty-nine percent of the patients had overexpression of Her2. Trends toward improved outcome for Her2-positive patients treated with the AC arm relative to the CMF arm were seen with an RR of relapse of 0.84 (95% CI = 0.65 to 1.07; *P* = .15) and with an RR for survival of 0.82 (95% CI = 0.63 to 1.06; *P* = .13). Trends toward a better outcome of the AC arm versus

the CMF arm were not seen for patients with erbB-2-negative tumors for DFS, with an RR of relapse of 1.02 (95% CI = 0.86 to 1.20; *P* = .84) or an RR for survival of 1.07 (95% CI = 0.88 to 1.30; *P* = .51). A borderline statistically significant interaction between doxorubicin treatment and erbB-2 overexpression was demonstrated for DFS (*P* = .19), with a trend for such an interaction demonstrated for survival (*P* = .11). The authors of this manuscript concluded that the results supported a preference for AC over CMF in patients whose tumors overexpress Her2.

Two Large Cooperative Group Studies Addressing This Issue

Analysis of Her2 in the European consortium trial of epirubicin-containing chemotherapy versus CMF. A European consortium has reported the results of their analyses (12,13) of the predictive value of Her2 as determined by IHC in a randomized phase III study in lymph node-positive patients comparing 1) CMF, 2) epirubicin (60 mg/m²)/cyclophosphamide (EC), and 3) epirubicin (100 mg/m²)/cyclophosphamide (HEC). The analysis of Her2 by IHC was performed only for the patients who were in the CMF and HEC arms. The median follow-up was 50 months. Samples from 481 patients were analyzed. Two different antibody cocktails were used. The first study used 4D5 and/or CB11, apparently on different samples. The second study used a mixture of antibodies similar to those used by the NSABP (a cocktail of the MAb-1 and the pAb-1 polyclonal serum). Cases were scored as positive if greater than or equal to 1% of the cells scored as positive. For the first and the second studies, 12% and 18% of the cases scored as positive, respectively.

The results were suggestive of a positive interaction between Her2 expression and anthracyclines for the first methodology but not for the second. The adjusted hazard ratios for the event-

free survival comparison of HEC versus CMF were as follows: Her-2 positive, 0.33 (95% CI = 0.09 to 1.27; $P = .08$); and Her-2 negative, 1.16 (95% CI = 0.71 to 1.90; $P = .56$). The P value of the interaction test was .10. When Her2 was evaluated by MAb-1 + pAb-1 antibodies, the adjusted hazard ratios for the same comparison were as follows: Her-2 positive, 1.06 (95% CI = 0.45 to 2.52; $P = .90$); and Her-2 negative, 0.99 (95% CI = 0.58 to 1.68; $P = .97$). The P value for the interaction test was .84. The authors rationalized the negative result with the second methodology as being as a result of 1) the low sample size and low statistical power and 2) the fact that the concordance of expression of Her2 by IHC and FISH (fluorescence *in situ* hybridization) was 92% for the first methodology but only 68% for the second. This suggests that the first methodology might be measuring something more tightly coupled to Her2 gene amplification than the second. Taken together, the results of this study are both encouraging (in one instance, the results supported the hypothesis) and discouraging. The results highlight that all Her2 methodologies are not equivalent and that some may be inferior and inadequate to be used to predict anthracycline sensitivity. It is particularly disappointing that the antibody cocktail used with success by the NSABP was not predictive in the hands of this second set of investigators.

Analysis of Her2 by the SWOG in specimens from adjuvant breast cancer trial S8814. Specimens (746 cases of the 1558 on study) have been analyzed from the adjuvant therapy trial S8814 (14). In this trial, postmenopausal estrogen receptor-positive women were randomly assigned to receive either tamoxifen for 5 years or chemohormonal therapy with six cycles of CAF and tamoxifen for 5 years (either starting it concurrently with the chemotherapy or after completion of the chemotherapy). For the purposes of the Her2 study, the treatment arms were collapsed to two: tamoxifen alone versus tamoxifen plus CAF.

IHC was performed in two separate studies using different IHC techniques. In the first study, MAb-1 (the same monoclonal antibody as that used by the NSABP) was used with the same interpretation of Her2 overexpression (any cells with definite membrane staining). This study showed clear trends for an interaction between Her2 overexpression and benefit from CAF. In this study, chemoendocrine therapy was superior to tamoxifen-alone therapy in the Her2-positive patients, with an RR for relapse of 0.53 (95% CI = 0.30 to 0.95; $P = .03$) and an RR for survival of 0.44 (95% CI = 0.24 to 0.83; $P = .009$). The chemoendocrine therapy arm was not superior in the patients whose tumors do not overexpress Her2, with RRs for DFS and overall survival (OS) of 0.85 and 0.96, respectively, neither of which reached statistical significance. In this underpowered study, the test for interaction between treatment and Her2 expression showed weak trends (0.24 and 0.10 for DFS and OS, respectively) but did not reach the level of conventional statistical significance.

In a second study using the monoclonal antibody CB11, no suggestion of predictive power for DFS or OS was seen (see Tables 2 and 3). In this study, there were no differences that reached statistical significance in the apparent efficacy of treatment with chemoendocrine therapy versus treatment with tamoxifen therapy in patients whose tumors either do or do not overexpress Her2. This is a disappointment, and in some ways it leads to the same conclusion as the European study—that details of the methodologies used are important.

Smaller Studies or Studies With Other Non-Idealities

There are studies with important weaknesses that have addressed the question of whether Her2 expression might predict the benefit of anthracycline-based therapy. A Spanish study (15) (with only 141 patients) that compared CMF with FAC found that FAC was superior to CMF in patients whose tumors overexpress Her2 but that FAC was equivalent to CMF in patients whose tumors do not overexpress Her2. This study is so small and underpowered as to not contribute strongly to the testing of the hypothesis. A small German study (16) with 144 patients randomly assigned to receive either high-intensity epirubicin or a more standard epirubicin dose intensity has reported on the effects of Her2 expression. In this study, both those patients whose tumors did and those patients whose tumors did not overexpress Her2 appeared to benefit from therapy, so the authors concluded that Her2 expression was not predictive of the benefit of dose intensification of an anthracycline. In reality, this study is so small as not to address this question with any real statistical power. An Italian study (17) (with 266 patients) that randomly assigned patients to receive either CMF or single-agent epirubicin had the same weakness of too few patients to have reasonable statistical power to investigate an interaction between treatment and Her2 expression. In this study, the DFS was equal in the two arms, with a trend toward epirubicin being more effective than CMF in patients whose tumors overexpress Her2 and less effective in the patients whose tumors do not overexpress Her2, but the study was so small that these trends did not reach statistical significance.

European Organization for Research and Treatment of Cancer (EORTC) trial 10854 compared the value of a single cycle of perioperative FAC to no further therapy. This study (18) made available Her2 determinations on 441 patients. Also, in this study, DFS was improved by FAC in the patients whose tumors do not overexpress Her2 (4-year DFS 85% versus 78%; $P = .05$) and also in the patients whose tumors overexpress Her2 (90% versus 77%; $P = .17$). The hazard of relapse seemed to be reduced to a greater extent in the patients with Her2-overexpressing tumors, but this study did not do a formal statistical test to demonstrate this interaction. This study, while in some ways supportive of the concept of an interaction between anthracycline responsiveness and Her2 expression, did not demonstrate this effect formally and, because of its design (a single perioperative cycle of therapy), may not be directly comparable with the studies of multiple cycles of therapy. A second analysis of these cases for these effects on local recurrence rates found no interaction between treatment effectiveness and Her2 expression, but this study (19) had so few events as to not have any power to demonstrate this interaction.

Of course, adjuvant phase II studies without a control group that received a non-anthracycline-based treatment plan cannot be used to address the question of the interaction between treatment effectiveness and Her2 expression because the effects of Her2 on prognosis cannot be distinguished from its effects on the efficacy of therapy (20,21).

CONCLUSIONS

Taken together, the evidence provides tantalizing but as yet inconclusive evidence that Her2 overexpression can be used to

identify patients with early breast cancer who will particularly benefit from anthracyclines. In the four major studies with at least some element of prospective character (the two NSABP studies, the European study, and the SWOG study), in most instances much (all?) of the apparent additional benefit of anthracyclines was seen in the subset of patients whose tumors overexpress Her2. Yet these studies demonstrate the inconclusive nature of the current evidence as well. In general, the studies are vastly underpowered, so effects that seem obvious to the eye do not reach statistical significance. In addition, the European and SWOG studies demonstrate the importance of the techniques used, with some Her2 IHC techniques seeming to be inferior methods for the detection of the interaction.

The road forward is obvious and, hopefully, will confirm the observation that anthracyclines seem to confer additional benefit in patients whose tumors overexpress Her2.

Better standardization of IHC Her2 methodologies among studies would seem important in the research studies. Ideally, methodologies should be used that have been approved for routine clinical evaluation of patient samples, so that the results, if truly predictive, can be brought quickly into clinical use with confidence in the methodology.

Whether other measures of Her2 have value for the prediction of treatment effects should be explored. For example, the use of FISH has led to better predictions than the use of IHC as to which patients are most likely to benefit from Herceptin. Perhaps FISH will be a better test for prediction of anthracycline sensitivity in adjuvant therapy.

Larger and better statistically powered studies are crucial; the ideal would be large studies based on large clinical trials testing anthracycline- and non-anthracycline-based adjuvant therapies. The value of meta-analyses in this field is problematic not only because of the variance in treatments among the trials but because of the multiplicity of the IHC methods used.

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Is HER-2/neu a Predictor of Anthracycline Utility? No.

George W. Sledge, Jr.

HER-2 has a well-established role as a prognostic indicator in breast cancer and as a predictor for response to trastuzumab. Recent studies have also suggested that it may serve as a predictor of response to anthracycline-based therapies. This article argues that the data are insufficient to accept this hypothesis as scientifically established. The argument is developed along several lines: first, that the trials used to support a predictive role for HER-2 have real flaws with regard to this hypothesis; second, that HER-2 is a remarkably inconsistent predictor of anthracycline response when examined in a broader context that includes preoperative and metastatic disease; third, that preclinical data fail to support the hypothesis; and finally, that even if accepted, the hypothesis is difficult to extrapolate to the everyday world of breast cancer. [J Natl Cancer Inst Monogr 2001; 30:85-7]

HER-2 is clearly important in the natural history of breast cancer. This importance derives from its role in tumor growth, invasion, and metastasis (the clinical summation of which is its role as a prognostic factor in early-stage breast cancer), as well as its role as a predictor of response to trastuzumab. These roles for HER-2 seem firmly established.

Recent U.S. cooperative group trials have suggested that patients receiving anthracycline-based chemotherapy are most likely to benefit if their tumors overexpress the HER-2 protein. This role of HER-2, as a predictor of anthracycline utility, seems promising and, if confirmed, clearly would be of importance. But to what extent should we accept the data presented to date?

I would like to suggest that what might be called the HER-2 hypothesis suffers from several potentially fatal flaws: 1) The data used to support the hypothesis are inherently flawed, as is the analysis of these data; 2) a broader view of the available data suggests that there are problems with consistency across the spectrum of breast cancer; 3) there is no solid biologic basis for the hypothesis; and 4) even if we were to accept the general hypothesis, practical extrapolation is difficult.

PROBLEMS WITH POSITIVE TRIALS

Several trials have reported positive results (1-3). Individually and collectively, these trials suffer from potential and actual flaws. All of the data presented to date (with the exception of a subset analysis of Cancer and Leukemia Group B [CALGB] 8541) (4) use immunohistochemical techniques, and such techniques are associated with well-known concerns. Immunohistochemical accuracy depends on proper tissue preservation, antigen retrieval, reagent specificity, type of technique used, protocol used for grading positivity, and observer experience. The ability of the antibodies used in immunohistochemical assays to detect HER-2 is highly variable, and the correlation of immunohistochemistry with the "gold standard" of fluorescence *in situ* hybridization is often relatively low. Similarly, immunohistochemical techniques often correlate poorly with each other (5,6).

All of these problems potentially come into play in the studies used to support the HER-2 hypothesis. In each study, tissue

collection was retrospective. In one of the studies (2), the antibody used was switched mid-study, and the correlation coefficient for the two antibodies used was 91%—good but not perfect. The same study evaluated interobserver reproducibility in the hands of two expert breast pathologists, with an overall $R^2 = 76\%$, a statistically significant result that is less than reassuring when one is dealing with potentially life and death decisions. In another, immunohistochemistry was done, in many cases, on old slides rather than on paraffin-embedded tissue (1). No two studies used the same scoring technique: In one study as few as 1% positive cells was used as a cutoff for positivity, whereas in another 50% positivity was used. One might interpret consistently positive results obtained from such different technical approaches as evidence of the strength of the overall hypothesis, yet it is equally reasonable to consider such studies as being essentially uninterpretable for comparative purposes.

Leaving aside the potential technical flaws inherent in immunohistochemical techniques, analysis of the reported trials leaves something to be desired. All involved retrospective, *post hoc* analyses and should, therefore, properly be viewed as hypothesis-generating rather than as proof-of-principal studies. In the CALGB trial, an initial analysis reported positive results, a second analysis with a somewhat larger set of patients failed to reach statistical significance, and a third analysis combining the two was once again positive. Because of an unbalanced randomization, the Southwest Oncology Group report involves analyses of truly small numbers of patients in the subset of HER-2-positive, estrogen receptor-positive patients. Tests for interaction did not reach statistical significance in any of the three trials with regard to overall survival, and for disease-free survival, only in the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial. As discussed by Ravdin in another article in this issue (7), the number of patients required to answer this question with any degree of scientific rigor is large, and it is larger than any current study affords.

Finally, what are we to make of the available trials themselves? It is tempting to view these trials through the lens of the HER-2 hypothesis, in effect to assume that each represents an attempt to test the HER-2 hypothesis. In reality, each trial tested a treatment hypothesis unrelated to the (*post hoc*) HER-2 hypothesis. As such, the best we can do (or should do) is to rephrase the HER-2 hypothesis in terms of the actual hypothesis tested in each protocol, yet to do so gives us some sense of the problems inherent in using these trials to support the HER-2 hypothesis.

For instance:

CALGB 8541. An increased dose intensity of combination chemotherapy with cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) (please note, *not* doxorubicin alone; all three agents were dose escalated) is beneficial in HER-2-positive patients; moderate-dose intensity CAF given for a longer duration

Correspondence to: George W. Sledge, Jr., M.D., Indiana Cancer Pavilion, RT-473, 575 Barnhill Dr., Indianapolis, IN 46202 (e-mail: gsledge@iupui.edu).
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(but with the same total dose as the high-dose arm) and low-dose intensity CAF are not beneficial in HER-2-positive patients.

NSABP B-11. Addition of low-dose, chronically administered doxorubicin to melphalan and 5-fluorouracil is beneficial in HER-2-positive but not in HER-2-negative patients.

Southwest Oncology Group 8814. CAF (please note, *not* doxorubicin alone) chemotherapy is beneficial in HER-2-positive, estrogen receptor-positive patients but has no benefit in HER-2-negative, estrogen receptor-positive patients.

It is important to note that only one of these trials (NSABP B-11) specifically evaluates the role of doxorubicin (as opposed to a doxorubicin-containing combination). Within the context of two of the trials above, it would be just as reasonable to assume that 5-fluorouracil or cyclophosphamide efficacy was modulated by HER-2 as was doxorubicin's efficacy. Those who support the HER-2 hypothesis have suggested that what is common to all three of these trials is that "more" doxorubicin always appears to work better than "less" doxorubicin in HER-2-positive patients, yet the low-dose-intensity arm of the CALGB trial uses essentially the same dose intensity as the doxorubicin-containing arm of NSABP B-11. How can the results of low-dose-intensity doxorubicin be strikingly ineffective in one trial, and yet so strikingly impressive in another?

One might argue that such cross-trial comparisons are inherently problematic, so that it is unfair to compare the results of these two similar low-dose-intensity arms. This is exactly the point. Those who lump together studies with such disparate primary hypotheses, drugs, schedules, and dose intensities for the purpose of supporting a *post hoc* hypothesis are guilty of exactly this practice. One would not accept the results of (or even contemplate performing) a meta-analysis of these very different trials in terms of their original hypotheses, yet, in effect, we are asked to perform an informal meta-analysis, a kind of science by gestalt, in a statistically less rigorous fashion in support of the HER-2 hypothesis.

PROBLEMS WITH CONSISTENCY

Although most doxorubicin-based adjuvant trials have been reported to be positive, at least one epirubicin-based analysis has been reported to show no differential benefit for HER-2 positivity. Untch et al. (8) compared a standard-dose epirubicin/cyclophosphamide combination with a dose-intense epirubicin/cyclophosphamide regimen. Only in the HER-2-negative subgroup was there a benefit for the dose-intense regimen, a result that contrasts strikingly with the CALGB results. Given that epirubicin is at least as beneficial in the adjuvant setting as doxorubicin, how could one explain the absence of HER-2-related benefit?

Similarly, Colozza et al. (9) evaluated the effect of HER-2 status in a trial comparing epirubicin with combination adjuvant chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil in patients with American Joint Committee on Cancer stage I or II breast cancer. With a median follow-up of 5.6 years and 266 patients evaluable for HER-2 status, epirubicin treatment had no statistically significant impact on the outcome of patients with HER-2-positive tumors.

One would expect a consistency of effect across all stages of breast cancer if the HER-2 relationship were real. We would not expect a predictive factor to have a benefit in stage II breast cancer but not in stage III or IV breast cancer, yet this is what we are asked to accept for the HER-2/doxorubicin interaction. Nu-

merous trials (10-19) have examined the effect of HER-2 overexpression on response to preoperative or metastatic anthracycline-based regimens. These trials (summarized in Table 1) are inconsistent in their results, but generally they fail to demonstrate a positive relation. While the number of *patients* entered in these trials is small relative to the adjuvant trials, the number of *events* is large, since virtually every patient entered is evaluable for response.

BIOLOGIC PROBLEMS

One would expect that, if HER-2 overexpression conferred sensitivity to doxorubicin in the clinic, a similar effect might be seen in the laboratory. Pegram et al. (20) tested this hypothesis by transfecting four breast cancer cell lines with HER-2 and then exposing them to doxorubicin *in vitro*. No alteration in chemosensitivity was observed in any of the transfected breast cancer cell lines compared with the parent cell lines or in a related *in vivo* nude mouse xenograft model. These observations argue against a direct role for HER-2 amplification in anthracycline sensitivity.

It has been suggested that HER-2's proposed relationship to anthracycline sensitivity might, in fact, be as a surrogate for topoisomerase II- α . Anthracyclines are, of course, topoisomerase inhibitors. Topoisomerase II- α is located close to HER-2 on chromosome 17, and recent evidence (14) suggests strongly that its amplification may be associated with sensitivity to anthracyclines in the metastatic setting. While this proposal seems reasonable, two observations may be made regarding it: 1) Jarvinen et al. (21) have examined the relation of HER-2 and topoisomerase II- α in patients with metastatic breast cancer. As it turns out, the two are not on the same amplicon, and when HER-2 is overexpressed, topoisomerase II- α is as often deleted as amplified. 2) Assuming that the explanation is correct, what benefit would be gained using a surrogate marker for topoisomerase II- α , when one could presumably measure the "real thing" with greater correlative power?

PROBLEMS WITH EXTRAPOLATION

The problems associated with accepting the results seen with the available adjuvant trials mirror the problems seen with

Table 1. Effect of HER-2 overexpression on response to anthracycline-based preoperative or metastatic chemotherapy regimens

Author	Stage	N	Effect*
Kling (16)	III	32	None
MacGrogan (15)	II or III	126	None
Vincent-Salomon (12)	II or III	54	None
Zapf (19)	III	46	None
Petit (23)	II or III	79	Negative (low dose) Positive (dose intensification)
Gregory (18)	II or III	283	Negative
Steger (25)	II or III	57	Positive
Niskonen (17)	IV	127	None
Jarvinen (10)	IV	55	Negative
Isola (14)	IV	196	None
Stender (13)	IV	311	None
Sjostrom (11)	IV	103	None
Rozan (24)	II or III	167	None

*Positive means that HER-2 patients are more likely to respond to chemotherapy; negative means that they are less likely to respond to chemotherapy, with a *P* value at least <.05. *N* = number of patients.

HER-2 testing in general. Immunohistochemical analysis of HER-2 is only imperfectly correlated with fluorescence *in situ* hybridization and is subject to considerable interobserver variation, problems with technique, and problems with tissue preservation. None of the current trials have even used the same immunohistochemical techniques. How then may we extrapolate the currently available results to clinically available testing kits?

Similarly, proponents of the HER-2 hypothesis should understand its logical practical conclusions. It is safe to guess that virtually all lymph node-positive, HER-2-positive patients currently receive doxorubicin-based therapy. Two thirds to three quarters of breast cancer patients are HER-2 negative. The same studies suggesting a benefit for doxorubicin in HER-2-positive patients suggest a relative lack of added benefit in HER-2-negative patients. Given the Oxford overview (22) demonstration of benefit for anthracycline-based regimens in the adjuvant setting, do we have sufficient confidence in the HER-2 hypothesis that we are willing to omit doxorubicin in HER-2-negative patients?

CONCLUSION

HER-2 testing has many real benefits. These benefits should not blind us to the real concerns surrounding the use of HER-2 as a therapeutic predictor for anthracyclines. We currently lack a solid biologic basis for the proposed linkage. The available positive studies have real uncertainties. There are studies contradicting the linkage in the adjuvant, neoadjuvant, and metastatic settings. Extrapolation from current data to the clinic is problematic. The cumulative weight of these concerns calls the HER-2 hypothesis into question. Until more solid data emerge, HER-2 positivity should not be accepted as a predictor of doxorubicin sensitivity.

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Taxanes in the Adjuvant Treatment of Breast Cancer: Why Not Yet?

Martine J. Piccart, Caroline Lohrisch, Luc Duchateau, Marc Buyse

The taxanes paclitaxel and docetaxel represent the most active chemotherapeutic agents developed for the treatment of advanced breast cancer in the last decade, and they are now being incorporated into adjuvant chemotherapy trials for lymph node-positive breast cancer with the hope of improving on the results achieved with CMF (cyclophosphamide, methotrexate, 5-fluorouracil) or anthracycline-based regimens. So far, three randomized paclitaxel-based adjuvant clinical trials enrolling 3170 women (Cancer and Leukemia Group B [CALGB] 9344), 3060 women (National Surgical Adjuvant Project for Breast and Bowel Cancers [NSABP]-B28), and 524 women (M. D. Anderson), respectively, have been reported with respective median follow-up times of 52, 34, and 43 months. This article critically reviews these three studies and gives an overview of the many other randomized clinical trials, due to accrue more than 17 000 women, which are investigating the potential of taxanes in adjuvant breast cancer therapy. Given that the early promise of taxanes suggested by CALGB 9344 is not yet confirmed by the two other trials, only level 2 evidence has been reached to date in regard to a positive contribution of these agents to breast cancer outcome in the adjuvant setting. It is argued that level 1 evidence is highly desirable before adopting taxane-based regimens in standard practice. It is anticipated that a meta-analysis will be needed to comprehensively define the value of taxanes in early breast cancer, and a new model of international collaboration is proposed to find a balance between the need to offer new, more effective therapies to patients as soon as possible and the danger of drawing wrong, premature conclusions regarding the magnitude of benefit of a new regimen. [*J Natl Cancer Inst Monogr* 2000;30:88-95]

INTRODUCTION

Adjuvant chemotherapy with CMF (cyclophosphamide, methotrexate, 5 fluorouracil) and anthracycline-based regimens is associated with significant reductions in breast cancer mortality (1). Research efforts have explored numerous treatment strategies in attempts to further improve survival, including the addition of newer anticancer drugs that have demonstrated activity in the metastatic setting, such as the taxanes.

Paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) and docetaxel (Taxotere; Aventis, Collegeville, PA) undoubtedly represent the most active chemotherapeutic agents developed in the last decade for the treatment of advanced breast cancer. Outstanding features of these agents that have been the focus of several reviews (2-5) include, first, their original mechanism of action, namely, binding to and stabilization of microtubules, thereby preventing their depolymerization; second, their partial lack of cross-resistance with anthracyclines, to which they compare favorably as far as single-agent activity; and third, their

capacity to be combined with almost all active chemotherapeutic agents commonly used for breast cancer therapy.

The next logical step in the clinical development of the taxanes was their incorporation into adjuvant chemotherapy regimens for lymph node-positive breast cancer with the hope of extending disease-free survival and overall survival.

The oldest of the two compounds, paclitaxel, was the first to enter the adjuvant scene; as a result, only paclitaxel-based adjuvant clinical trials have been reported to date. Fig. 1 summarizes the chronology of those reports and their affect on regulatory agencies in the United States (U.S. Food and Drug Administration [FDA]) and in Europe (European Medical Agency [EMA]).

The large U.S. Intergroup trial, referred to as Cancer and Leukemia Group B (CALGB) 9344, explored the value of adding four cycles of paclitaxel to four cycles of AC (doxorubicin-cyclophosphamide) as postoperative adjuvant therapy of lymph node-positive breast cancer in 3170 women. This trial, in fact, had a 3×2 factorial design to compare three doses of doxorubicin (60, 75, or 90 mg/m² by random allocation) plus cyclophosphamide (600 mg/m²) given intravenously on day 1 every 3 weeks for four courses (AC \times 4), and then, second, to compare paclitaxel at a dose of 175 mg/m² as a 3-hour infusion every 3 weeks given for four courses following AC (AC/T) versus no additional chemotherapy. The superior results of the paclitaxel arm were reported at its first planned interim analysis, conducted at a median follow-up time of 20 months, and were presented at the 1998 meeting of the American Society of Clinical Oncology (ASCO) (6); an update with a median follow-up of 30 months was presented to the ODAC (Oncology Drug Advisory Committee) with subsequent registration of the paclitaxel-based adjuvant regimen for lymph node-positive disease by the FDA in late 1999.

In contrast, in March 2000, the European Regulatory Agency, provided with the same set of data (7), viewed these data as "premature" and did not license paclitaxel for adjuvant use in the European Union. Since then, two other paclitaxel-based adjuvant breast cancer trials have been reported with inconclusive results: the relatively small M. D. Anderson trial presented at the 2000 meeting of ASCO (8) and the large National Surgical Adjuvant Project for Breast and Bowel Cancers (NSABP)-B28 trial presented at the November 2000 NIH Breast Cancer Consensus Conference (9).

In the former trial, women were randomly assigned to receive either eight cycles of FAC (5-fluorouracil, doxorubicin, and cy-

Affiliations of authors: M. J. Piccart, C. Lohrisch, Jules Bordet Institute, Brussels, Belgium; L. Duchateau, European Organization for Research on Treatment of Cancer—Data Center, Brussels; M. Buyse, International Institute for Drug Development, Brussels.

Correspondence to: Martine J. Piccart, M.D., Ph.D., Jules Bordet Institute, 1 Rue Héger-Bordet, B-1000 Brussels, Belgium (e-mail: martine.piccart@bordet.be).

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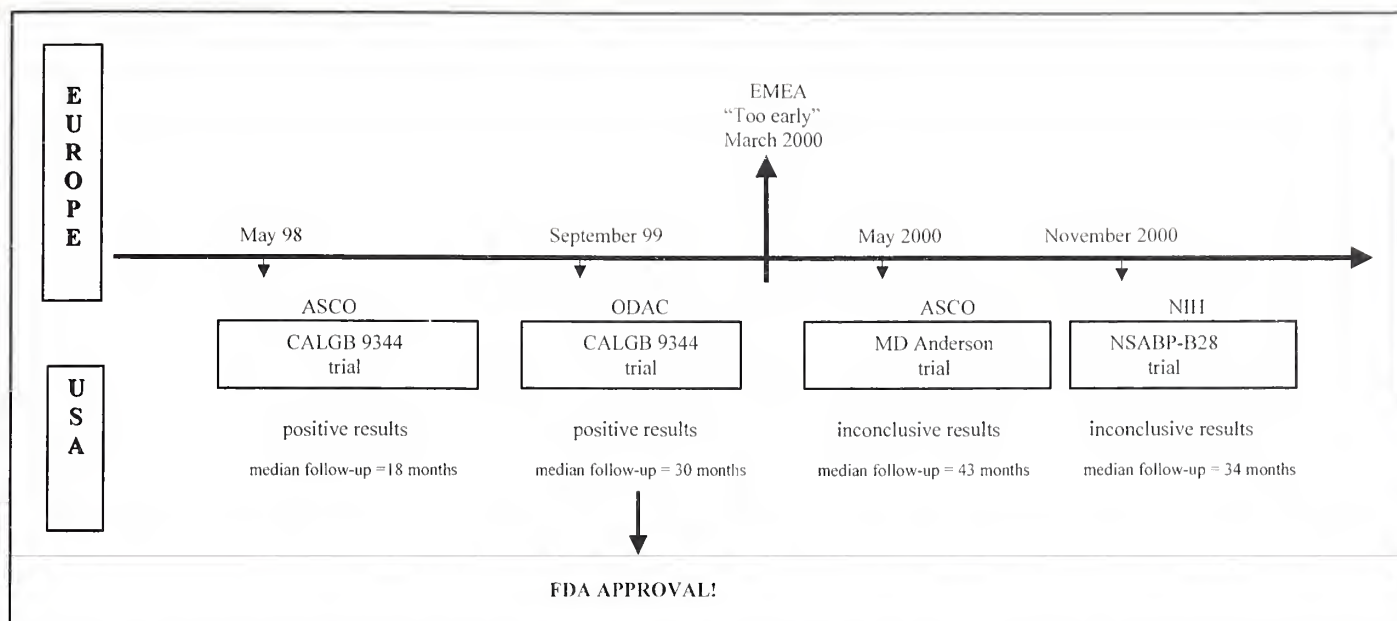


Fig. 1. Paclitaxel (Taxol) in breast cancer adjuvant therapy: chronology of events. ASCO = American Society of Clinical Oncology Meeting; CALGB = Cancer and Leukemia Group B; EMEA = European Medicine Evaluation Agency; FDA = Food and Drug Administration; ODAC = Oncology Drug Advisory Committee.

clophosphamide) or four cycles of paclitaxel followed by four cycles of FAC, with a proportion of patients receiving some of the chemotherapy preoperatively. Although there was a 24% risk reduction for recurrence in the group treated with paclitaxel, this was not significant, given the short follow-up and small trial size. No difference in overall survival was observed between the two groups.

The NSABP-B28 trial, conducted in 3060 women with lymph node-positive breast cancer, adopted a very similar design to the one of CALGB 9344, with four cycles of AC being compared with four cycles of AC followed by four cycles of paclitaxel, whereas the former trial randomly assigned 524 patients to eight cycles of FAC or four cycles of paclitaxel followed by four cycles of FAC.

In this article, we carefully review the available evidence that led us to conclude that taxane-based regimens cannot at present be recommended as gold-standard adjuvant chemotherapy for women with invasive breast cancer.

SIMILARITIES AND DIFFERENCES BETWEEN THE THREE REPORTED PACLITAXEL TRIALS: CALGB 9344, M. D. ANDERSON, AND NSABP-B28

Treatment Differences

Both CALGB 9344 and NSABP-B28 opted for a relatively short anthracycline-based control arm (four cycles of AC), whereas the M. D. Anderson trial chose eight cycles of FAC as control. The dose and sequence of chemotherapy regimens varied in the three trials: Only CALGB 9344 intensified the dose per cycle of anthracycline compared with a standard AC, keeping the total number of cycles at four. The NSABP-B28 study used a standard AC regimen with respect to anthracycline dose but gave a higher paclitaxel dose: 225 mg/m², as opposed to 175 mg/m² in CALGB 9344. In the M. D. Anderson trial, paclitaxel was given before anthracyclines and as a 24-hour infusion, and the anthracycline regimen consisted of eight cycles in the control arm, compared with four cycles in both CALGB

9344 and NSABP-B28. This last trial was the only one of the three trials to have the same total number of cycles in both the paclitaxel and nonpaclitaxel arms.

Also, while patients in both CALGB 9344 and NSABP-B28 received all chemotherapy postoperatively, this was only the case in two-thirds of the patients in the M. D. Anderson trial; the remainder received four courses before and four courses after primary surgery.

All three trials planned adjuvant tamoxifen therapy for patients with hormone-sensitive tumors, defined as those having estrogen receptor (ER)-positive and/or progesterone receptor (PgR)-positive tumors. In addition, all women more than 50 years old were given adjuvant tamoxifen in NSABP-B28 irrespective of their hormone receptor status. As a result of these different criteria, 85% of the women received tamoxifen in NSABP-B28, compared with only 70% in CALGB 9344.

Finally, the timing of adjuvant tamoxifen differed: It was given at completion of chemotherapy in the CALGB and M. D. Anderson studies but given concomitantly with chemotherapy in NSABP-B28.

Patient Population Differences

It is worth noting that CALGB 9344 enrolled patients at a higher risk of relapse than did NSABP-B28: Slightly more than half of the trial population had four or more positive axillary lymph nodes in the former, as compared with approximately one-third in the latter (6).

In the M. D. Anderson trial, the proportion of patients with zero, one to three, and four or more positive lymph nodes was approximately one third each (8).

Finally, NSABP-B28 seems to have recruited a somewhat older patient population, with 51% of the women being under the age of 50 years, compared with 56% of the women being under 50 years of age in the M. D. Anderson trial and a total of 62% premenopausal women in CALGB 9344. The NSABP trial also had the highest rate of hormone receptor-positive tumors (66% versus 58% in the two other studies) (9).

Outcome of the Trials

CALGB 9344 is now quite mature, with a median follow-up of 52 months and 901 events, including 589 deaths. As with its first planned interim analysis (presented in May 1998 after the occurrence of 453 events) and its second unplanned interim analysis (presented at the FDA in September 1999 with 624 events), results continue to show both disease-free and overall survival advantages for the AC/T arm, with a relative risk reduction for recurrence of 13% and for death of 14% compared with the AC-alone arm (10). This trend toward decreased mortality in the AC/T compared with AC arms no longer reaches statistical significance, although the disease-free survival remains significantly better for AC/T. A possible explanation is the emergence of late recurrences: The addition of paclitaxel may have reduced early recurrences in aggressive tumors, for which four cycles of AC were inadequate, but a second peak of late recurrences of these and of less aggressive tumors, on which the addition of paclitaxel did not have a major effect, is resulting in a narrowing of the treatment effect for AC/T.

The small M. D. Anderson trial, with 75 events and 28 deaths, does not show an advantage for the paclitaxel arm at a median follow-up of 43 months.

The third interim analysis of the NSABP-B28 trial, with 551 events and 269 deaths, does not show a difference either, with a median follow-up of 34 months (the first two interim analyses were not reported since they showed no significant treatment effect). The estimated overall survival at 36 months is 92% for the AC arm and 90% for the AC/T arm; estimated disease-free survival at 36 months is 81% in both arms (9).

DISCUSSION

Weaknesses of CALGB 9344

The CALGB 9344 trial has design limitations, and it is unclear to what extent these explain the apparent paclitaxel effect. A major potential confounder in this trial is the duration of therapy, which is longer in the paclitaxel-containing arm by 12 weeks (four cycles of cytotoxic therapy). This issue represents a difficult puzzle because the trials available to date do not

adequately answer the question of the relative importance of total duration of therapy and cumulative dose.

The Oxford overview reported that anthracycline-based therapy may be associated with a small survival advantage over CMF (1). Whereas studies (6,11,12) have failed to demonstrate that intensification beyond a threshold dose intensity improves survival, below that threshold there does seem to be a dose-response relationship, with a compromised outcome (13,14). Thus, we are faced with the question of whether the cumulative dose of doxorubicin (A) in four cycles of AC (240 mg/m²) is below this threshold, rendering it equally effective to CMF but less effective than it could be. If the answer is yes, it may explain the apparent discrepancy in the results of NSABP-B15 (equivalence for six cycles of CMF and four cycles of AC 240 mg/m² doxorubicin total dose), the National Cancer Institute of Canada comparison of CEF (cyclophosphamide, epirubicin, 5-fluorouracil) and CMF (CEF 7% superior overall survival, 720 mg/m² epirubicin total dose), and the Intergroup trial (INT 0102) of CAF (cyclophosphamide, Adriamycin, 5-fluorouracil) versus CMF (CAF 2% superior overall survival) (15-17). Indeed, although four cycles of AC (doxorubicin at 60 mg/m² per cycle) is a standard adjuvant regimen in North America, many European and Canadian oncologists give several cycles of CMF following four cycles of AC or four cycles of A or a higher total dose of anthracyclines, such as can be found in CAF/FAC and CEF/FEC regimens.

In the case of this study, both the different duration of chemotherapy in the paclitaxel and nonpaclitaxel arms and the potentially inadequate (or suboptimal) anthracycline cumulative dose in one-third of the patients treated in the control arm may have biased the results in favor of the AC/T arm.

Table 1 summarizes the few randomized trials that have compared 6 months of CMF with 3 months of CMF or 6 cycles of anthracycline-based therapy with three cycles of anthracycline-based therapy in operable breast cancer patients with positive axillary lymph nodes.

The first two trials (18,19), described in Table 1, were conducted in Germany, enrolled a majority of postmenopausal women, and used an intravenous day 1 + 8 CMF regimen. Each accrued fewer than 1000 women and failed to detect an advan-

Table 1. Duration of adjuvant chemotherapy and outcome: randomized trials of 6 months' treatment versus shorter treatment in lymph node-positive breast cancer*

Group (reference No.)	Comparison	No. of eligible patients	Patients who are estrogen receptor negative, %	Patients who are premenopausal or aged <50 y, %	Follow-up, y	Results, DFS (HR longer vs. shorter)	Results, OS
GBSG (18)	6 CMF (iv d 1 + 8) 3 CMF ± tamoxifen	473	≈30	42	9	0.95 95% CI = 0.74 to 1.2	
GABG (ASCO) (19)	6 CMF (iv d 1 + 8) 3 CMF	789 (-122)†	≈66	15	3	No difference (P = .34)	
IBCSG (VI) (20)	6 CMF 3 CMF ± reintroduction	1475	≈30	100	5	58 53 P = .04	No difference
FASG (ASCO) (21)	6 FE (50) C 3 FE (50) C 3 FE (75) C	602	≈27	100	8	55 46 47 P = .04 (P = .01)	67 61 64 (P = .06)

*GBSG = German Breast Cancer Study Group; GABG = German Adjuvant Breast Cancer Study Group; IBCSG = International Breast Cancer Study Group; FASG = French Adjuvant Study Group; ASCO = American Society of Clinical Oncology; C = cyclophosphamide; E = epirubicin; F = 5-fluorouracil; M = methotrexate; CI = confidence interval; DFS = disease-free survival; HR = hazard ratio; OS = overall survival; iv = intravenous; d = day.

†122 patients excluded as ineligible.

tage for the longer CMF treatment at median follow-up times of 9 and 3 years, respectively.

In contrast, the larger IBCSG (International Breast Cancer Study Group) trial VI (20) used the classic oral CMF regimen and targeted exclusively premenopausal women, one-third of whom had ER-negative tumors. At a median follow-up of 5 years, disease-free-survival, but not overall survival, was significantly better for the 6-month CMF arm than for the 3-month CMF arm, with a subset analysis suggesting that this benefit was more evident for younger women (aged <40 years) and for those with ER-negative tumors.

The last study, conducted in France, also focused exclusively on premenopausal women, comparing six cycles of FEC given once every 3 weeks (5-fluorouracil, epirubicin at a dose of 50 mg/m², and cyclophosphamide) with three cycles of either the same regimen or a regimen with a somewhat higher dose of epirubicin (75 mg/m²). The results of this trial, recently updated with an 8-year median follow-up (21), show that six cycles of FEC are superior to three cycles of FEC as far as disease-free survival, with a similar trend for overall survival. The weakness here is the suboptimal epirubicin dose per cycle, which prohibits comparison of these data to those obtained using adequately dosed anthracycline-based regimens.

We can tentatively draw three conclusions: 1) that the issue of the optimal duration of adjuvant chemotherapy has not been adequately studied; 2) that two clinical trials targeting premenopausal, lymph node-positive breast cancer patients have shown that 3 months or three cycles of adjuvant chemotherapy are inferior to 6 months or six cycles of adjuvant chemotherapy; and 3) that a subset analysis in one of these trials suggests that the ER-negative tumors benefit from longer treatment.

These three conclusions raise the concern that an increased duration of chemotherapy for an ER-negative population, in particular, may have contributed to the paclitaxel effect, a concern that is reinforced by the results of a subset analysis of CALGB 9344, which strongly suggests that only patients with hormone receptor (HR)-negative tumors (one-third of the study population) benefit from the addition of paclitaxel.

For the 2066 HR-positive patients, the hazard ratio for recurrence was 0.92 (95% confidence interval [CI] = 0.73 to 1.16) for AC/T versus AC, whereas for HR-negative patients, it was 0.68 (95% CI = 0.55 to 0.85) (7). A similar trend was observed in the M. D. Anderson trial: 58% of the population was HR positive, and although not statistically significant, the absolute difference in disease-free survival for FAC versus paclitaxel/FAC was 3% for HR-positive patients and 5% for HR-negative patients.

There are several potential explanations for the lower event rate in the AC/T arm to date: First, the baseline risk for patients with HR-positive tumors is lower and, therefore, a benefit of paclitaxel is more difficult to demonstrate, particularly if it is small; or, second, the baseline risk of HR-positive patients is sufficiently lowered by AC and tamoxifen that the added benefit of paclitaxel, if it exists, cannot be demonstrated with this sample size and follow-up period; or, third, recurrences in the HR-positive population occur later and a benefit may still become apparent with longer follow-up.

In the NSABP-B28 trial, a subset analysis did not suggest a clear benefit of adding paclitaxel in the small subset of patients who did not receive tamoxifen (9). Given the apparent relationship between HR status and benefit suggested by the CALGB

9344 and M. D. Anderson trials, an analysis according to ER status would be of interest in this trial, as well.

It is important, however, to remember that subset analyses are dangerous and potentially misleading: The trials that have compared 5-fluorouracil plus levamisole with no chemotherapy in colorectal cancer nicely illustrate how two trials of similar design and similar size can draw opposite conclusions as to who benefits, based on subset analysis.

The trial by Moertel and colleagues (22,23) concluded that the regimen was effective in Dukes' C but not Dukes' B colorectal cancer, while the one by Zoetmulder et al. (24) stated that the therapy provided benefit to Dukes' C and B colon cancer patients but not to those with rectal cancer.

Nevertheless, subset analyses generate interesting hypotheses, and the CALGB 9344 analysis by ER status, even if unplanned *a priori*, raises awareness about potential population differences in the magnitude of the taxane benefit, if such a benefit can be confirmed by other trials. In any event, these analyses support the contention that we cannot make sweeping generalizations about the value of adjuvant paclitaxel on the strength of the available evidence.

Table 2 illustrates the design of four trials ready to start or still being discussed in Europe and/or Canada that challenge CALGB 9344 on several important issues: first, a potentially improved anthracycline-based control arm given for 6 months (MA21 trial, U.K. trial); second, a dose-dense epirubicin-cyclophosphamide (MA-21 trial) or an individually "targeted" FEC regimen (Scandinavian trial) as opposed to higher fixed doses of doxorubicin given in combination with cyclophosphamide every 3 weeks (CALGB 9344); third, other nontaxane sequential regimens following four epirubicin cycles (U.K. trial); and, last but not least, the key issue of which subset of patients might derive the greatest benefit from adjuvant taxane-based therapy (Breast International Group [BIG]-01-00 study).

This last trial, to be coordinated by the EORTC (European Organization for Research and Treatment of Cancer) under the umbrella of the BIG (25), is a trial of considerable interest today, given the present confusion regarding the role of the taxanes in adjuvant breast cancer therapy. It is powered to test a biologic hypothesis, namely, that the benefit of taxanes might be confined largely to the subgroup of patients with p53-mutated tumors. This hypothesis has more support from laboratory data than the one relating taxane benefit to hormonal receptor status (26-33). Eligibility requirements for this trial include availability of an adequate tumor biopsy, part of which will be snap-frozen and processed for a p53 functional yeast assay and for microarray analysis.

The last concern raised by CALGB 9344, before the third analysis presented by C. Henderson, is the appropriateness of presenting positive interim results early, especially if the level of significance is not corrected for the fact that they are interim analyses. The initial results were reported less than 1 year after the accrual of the last patient (3-year accrual period) based on a preplanned interim analysis at the time of 450 events. One cannot extrapolate these significant results to later points in time unless one assumes a constant hazard ratio over time (proportional hazards model). Given that there appear to be two peaks of breast cancer recurrence, at 2 and 5 years, it is likely that this assumption is incorrect.

Clinical trial statisticians today perform planned interim analysis based on a precalculated number of events, as this tech-

Table 2. Planned or ongoing randomized clinical trials that challenge Cancer and Leukemia Group B 9344*

Group	Trial	Trial design	Comments
NCI-C-CTG	MA-21	6 CEF (Canadian) 4 AC → 4 P Dose-dense 6 EC → 4 P + G-CSF + erythropoietin	Same treatment duration in all three arms Hypothesis: I = II, III>I, or II
UK-CRC	TACT	8 FEC 4 FEC → 4 D 4 E → 4 CMF	Same treatment duration in all three arms Trial still under discussion
Scandinavian trial	SBG01-XX	8 Tailored FEC 4 AC → 4 P	Tailored FEC means increasing doses in the individual patient up to a target degree of myelosuppression
BIG (coordinated by EORTC)	BIG 01-00	FEC or Canadian CEF (preop) 3 D → 3 EC Frozen biopsy for p53 analysis mandatory	Hypothesis: D increases disease-free survival by 20% in p53+ tumors but only by 5% in p53 p- tumors

*NCI-C-CTG = National Cancer Institute of Canada Clinical Trials Group; UK-CRC = United Kingdom-Cancer Research Campaign; BIG = Breast International Group; EORTC = European Organization for Research and Treatment of Cancer; A = adriamycin; C = cyclophosphamide; D = docetaxel; E = epidoxorubicin; F = 5-fluorouracil; M = methotrexate; P = paclitaxel; G-CSF = granulocyte colony-stimulating factor; Preop = preoperatively; Dose-dense = every 2 weeks; Canadian CEF = cyclophosphamide 75 mg/m² orally for 14 days, epidoxorubicin 60 mg/m² intravenously day 1 + 8, 5-fluorouracil 500 mg/m² intravenously day 1 + 8, administered every 4 weeks with prophylactic antibiotics.

nique is most sensitive in detecting early treatment effects. Moreover, it is most often an Independent Data Monitoring Committee that decides whether interim results should be reported, based on the best interest of our patients and as a useful indication for future clinical research programs, but this decision should be motivated by the statistical interim analysis plan that describes at what level a planned interim analysis shows a significant treatment effect. Whatever the method used for this planned interim analysis (34–36), however, none of them would declare the *P* value of .039 significant with an overall nominal significance level of 5%. Such interim results are too statistically unstable to be trusted, and unadjusted *P* values may lead clinicians to come to the incorrect conclusion that the effect of treatment is statistically significant. Generally accepted guidelines for reporting interim results of clinical trials clearly mandate that the procedures adopted should always ensure that the overall probability of a false-positive error is controlled (37). Furthermore, such preliminary statements about treatment effects may influence the further results of the trial. Even worse, these early published results might jeopardize further accrual in trials that are still open to patient enrollment.

The real controversial issue here is whether or not, in a rapidly moving research field that brings to the clinic dozens of interesting new anticancer agents, early results of one large trial should lead to new standards of care.

Are the M. D. Anderson and the NSABP-B28 Trials Negative Trials?

The M. D. Anderson and NSABP-B28 trials are still compatible with a small absolute benefit from adjuvant paclitaxel (perhaps a 1%–2% gain in overall survival) that may be presently undetectable but that could emerge with longer follow-up. This is a reasonable assumption for NSABP-B28 because this trial has many patients and, therefore, has adequate power to detect a small but clinically relevant benefit. The M. D. Anderson trial, however, is a smallish trial for this adjuvant setting and, therefore, will only ever show significance for a large treatment benefit.

Therefore, these two trials presently neither help nor hinder the case for adjuvant paclitaxel, which means that we are left

both with one large positive trial showing divergent effect across HR subpopulations and with two inconclusive trials.

Balancing New Effective Therapies With Premature Reporting of Their Potential Benefits

A balance is needed between being able to offer new effective therapies as soon as possible and the danger of presenting premature and possibly incorrect conclusions about the magnitude of benefit of such new therapies. At present, we have only reached level 2—evidence that taxanes contribute some additional benefit in breast cancer adjuvant therapy, given one positive trial and two inconclusive ones (Table 3) (38). Level 1 evidence is highly desirable for reaching a consensus worldwide but is unlikely to be obtained without a meta-analysis, given the high probability that some trials will show an effect and others will not.

Changing clinical practice on the basis of one trial causes confusion and can be detrimental when subsequent trials fail to confirm the findings of the first. The surprising and as yet unexplained story of paclitaxel in the front-line treatment of advanced epithelial ovarian cancer is worth summarizing here because it illustrates this confusion particularly well. Two consecutive randomized clinical trials—the GOG (Gynecologic Oncology Group) #111 trial and the European-Canadian intergroup trial—addressing the question of a potential superiority of a paclitaxel–cisplatin regimen over a cyclophosphamide–cisplatin regimen showed remarkable similarities in outcome, making the medical community on both sides of the Atlantic confident that a new gold-standard chemotherapy regimen, namely paclitaxel–cisplatin, was born in the year 2000 (39,40). Unfortunately, a few months later, the results of a much larger multicenter trial,

Table 3. Types of evidence levels that form the basis of treatment options

Level 1 evidence	More than one consistent randomized clinical trial and/or meta-analysis
Level 2 evidence	One or a few unconfirmed randomized trials or more randomized trials with conflicting results
Level 3 evidence	Evidence from nonrandomized trial with reliable external control

the Third International Collaborative Ovarian Neoplasm Study (ICON3), comparing carboplatin–paclitaxel with carboplatin or CAP (cyclophosphamide, doxorubicin, cisplatin) were presented along with a 29-month median follow-up and showed no apparent overall advantage for the paclitaxel-containing arm (41). It is unclear whether the disappointing ICON3 results will change with a longer follow-up, but they certainly call for a meta-analysis of all trials, published or not, that have looked at potential improvement from taxane incorporation into first-line chemotherapy for epithelial ovarian cancer.

Fig. 2 summarizes all reported and unreported taxane adjuvant breast cancer trials in the world (42) and shows the huge imbalance between the reported trials, which have enrolled 6754 women, and the unreported ones, which are accruing about 17 854 women. Among the latter, four trials that are all exploring the potential benefit of adjuvant docetaxel (Taxotere) are already closed: They have accrued 8700 women. The remaining seven trials, which explore the potential benefits of either paclitaxel or docetaxel, are ongoing with a planned total enrollment of 9150 women. Waiting until these trials are completed and published before conducting a first meta-analysis would mean a long delay before settling the question of the effect of adjuvant taxanes.

In an ideal world, one could envisage setting up a large, independent meta-analysis unit that would have the capacity to respond to today's rapid acceleration of adjuvant trials conducted across continents. If it were provided with data on events occurring in all recently closed trials, examining a similar question (in our case, the taxane contribution to outcome in adjuvant breast cancer therapy) at regular intervals, the magnitude of

therapeutic effect of the test question could be estimated earlier and with more precision.

This model, illustrated in Fig. 3, is very challenging because it implies strong collaboration between groups involved in trials of adjuvant therapy as well as mutual trust and close contact with independent statisticians. It could be designed in a way that would prevent any public disclosure of individual trial results, the main emphasis being the periodic evaluation of the overall treatment effect and its statistical reliability. Such a model would probably represent a gain of several years in reaching enough confidence in the contribution of a particular agent or regimen to improved patient outcome. It is, therefore, a desirable model and places the interest of patients above the interests of investigators or the pharmaceutical industry.

The Future of Adjuvant Taxane-Based Therapy

With more than 20 000 women enrolled in trials exploring the potential benefit of taxane incorporation into adjuvant chemotherapy programs (42), one can be confident that their potential contribution to improved survival, even if modest, will be identified by a well-conducted overview. This overview should explore differential treatment effects in different patient subsets, defined by treatment, patient, or even tumor molecular marker characteristics whenever available.

Regarding treatment strategies, it is important to acknowledge that, thus far, available data have come only from trials giving paclitaxel- and anthracycline-based combinations in sequence. Table 4 shows that at least seven trials are exploring the potential added value of taxanes given in combina-

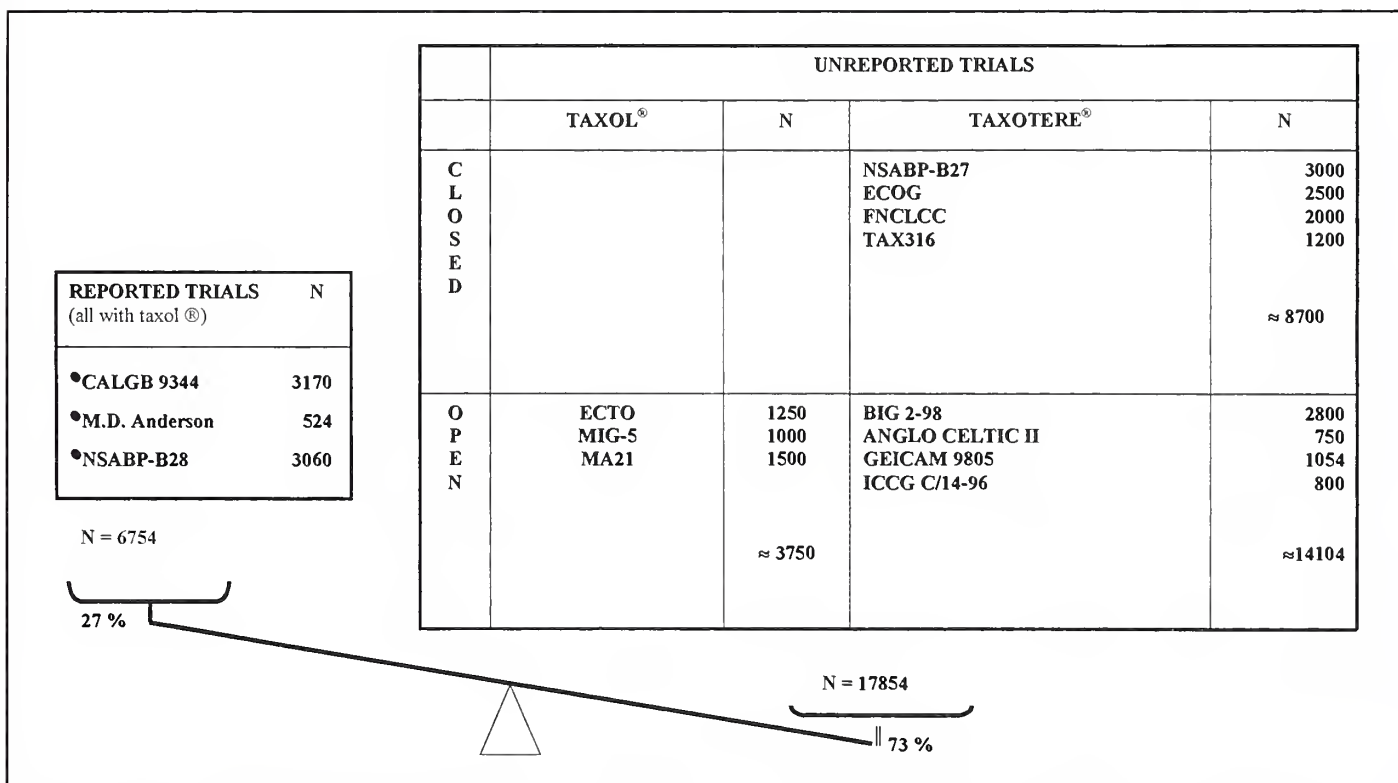


Fig. 2. Taxane-based adjuvant breast cancer trials worldwide. Taxol = paclitaxel; CALGB 9344 = Cancer and Leukemia Group B; NSABP-B28 = National Surgical Adjuvant Breast and Bowel Project; ECTO = European Cooperative Trial in Operable Breast Cancer; MIG-5 = Gruppo Oncologico Nord Ovest; Taxotere = docetaxel; ECOG = Eastern Cooperative Oncology Group; FNCLCC = Fédération Nationale des Centres de Lutte contre le cancer; BIG = Breast International Group; ANGLO CELTIC = Anglo Celtic group; GEICAM = Grupo Espanol de Investigacion en Cancer de Mama; ICCG = International Collaborative Cancer Group.

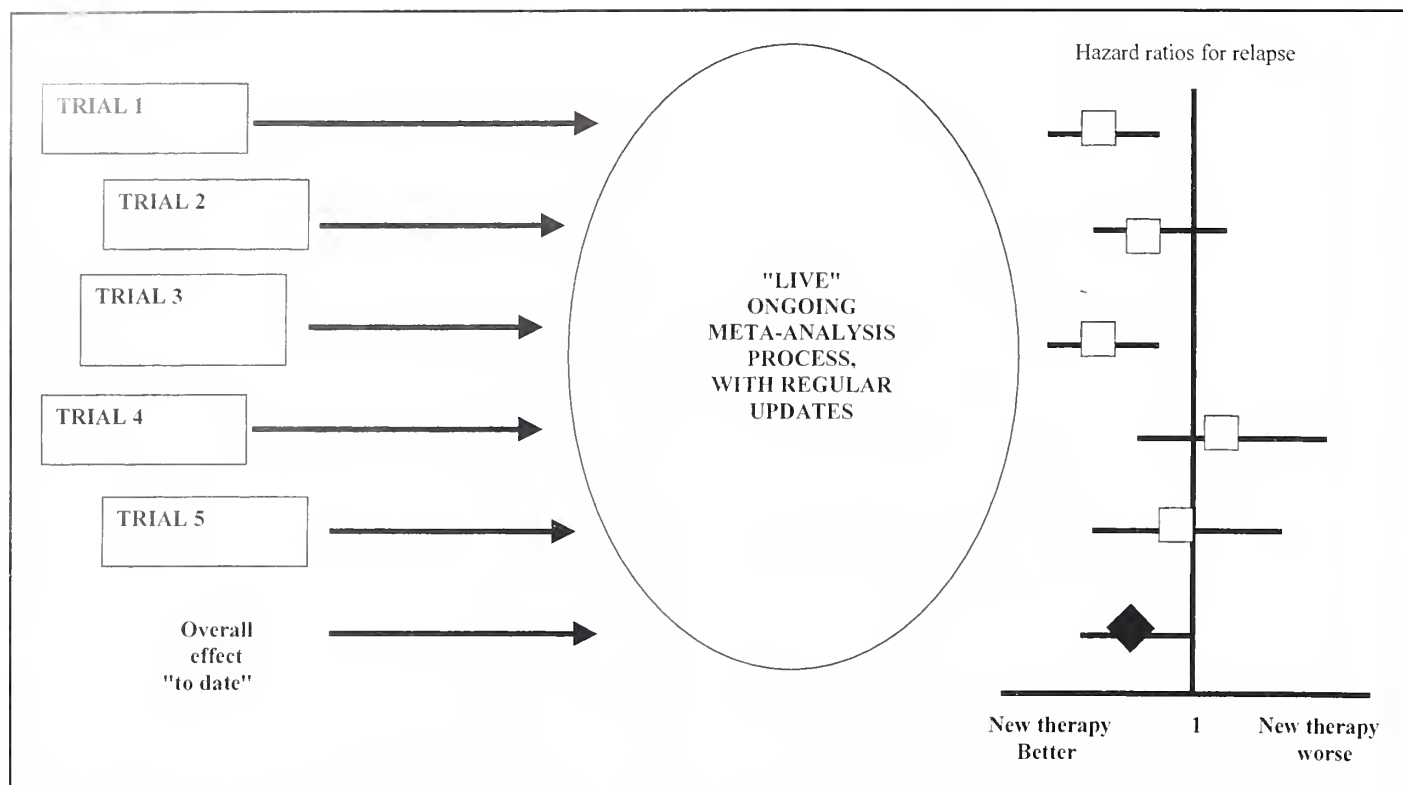


Fig. 3. Adjuvant breast cancer therapy in an ideal world. Trials 1 to 5 = Trials investigating a similar question, closed to patient entry.

Table 4. Complementary designs of taxane-based adjuvant breast cancer trials worldwide*

	Taxanes in combination (no asymmetry in duration)	Taxanes in sequence (no asymmetry in duration)	Sequence or combination: which is best?
Taxol	4 ET vs. 6 CEF (MIG-5) 4 AT → 4 CMF vs. 4 A → 4 CMF 4 (ECTO)		8 ET vs. 4 EC → 4 T
Taxotere	TAC vs. FAC (TAX316, GEICAM) AT vs. AC (ECOG, ANGLO-CELTIC)	3 FEC → 3 T vs. 6 FEC (FNCLCC) 3 E d1 + 8 → 3 T vs. E 6 d1 + 8 (ICCG)	4 A → 3 CMF vs. 3 A → 3 T → 3 CMF vs. 4 AT → 3 CMF vs. 4 AC → 3 CMF (BIG 2-98)

*A = Adriamycin, C = cyclophosphamide, E = epidriamycin, F = 5-fluorouracil, M = methotrexate, T = either Taxol or Taxotere, ANGLO CELTIC = Anglo Celtic Group, BIG = Breast International Group, ECOG = Eastern Cooperative Trial in Operable Breast Cancer, ECTO = European Cooperative Trial in Operable Breast Cancer, FNCLCC = Fédération Nationale des Centres de Lutte contre le Cancer, GEICAM = Grupo Español de Investigación en Cáncer de Mama, ICCG = International Collaborative Cancer Group, MIG-5 = Gruppo Oncologico Nord Ovest-Mammella Intergruppo, TAX316 = Aventis study number 316 with docetaxel.

tion with anthracyclines. One trial, BIG 2-98, is directly comparing the combination strategy; in this case, it is docetaxel combined with doxorubicin, with the sequential strategy, namely, single-agent docetaxel following single-agent doxorubicin (42).

It is very rewarding to see the tremendous international collaboration that is making these large trials possible. Let us hope that the learning curve in the conduct of these intergroup trials will soon be accompanied by innovative and creative ways to more rapidly and more efficiently collect and analyze the data these trials are generating.

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Preoperative Chemotherapy in Patients With Operable Breast Cancer: Nine-Year Results From National Surgical Adjuvant Breast and Bowel Project B-18

Norman Wolmark, Jiping Wang, Eleftherios Mamounas, John Bryant, Bernard Fisher

National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol B-18 was initiated in 1988 to determine whether four cycles of doxorubicin/cyclophosphamide given preoperatively improve survival and disease-free survival (DFS) when compared with the same chemotherapy given postoperatively. Secondary aims included the evaluation of preoperative chemotherapy in downstaging the primary breast tumor and involved axillary lymph nodes, the comparison of lumpectomy rates and rates of ipsilateral breast tumor recurrence (IBTR) in the two treatment groups, and the assessment of the correlation between primary tumor response and outcome. Initially published findings were based on a follow-up of 5 years; this report updates results through 9 years of follow-up. There continue to be no statistically significant overall differences in survival or DFS between the two treatment groups. Survival at 9 years is 70% in the postoperative group and 69% in the preoperative group ($P = .80$). DFS is 53% in postoperative patients and 55% in preoperative patients ($P = .50$). A statistically significant correlation persists between primary tumor response and outcome, and this correlation has become statistically stronger with longer follow-up. Patients assigned to preoperative chemotherapy received notably more lumpectomies than postoperative patients, especially among patients with tumors greater than 5 cm at study entry. Although the rate of IBTR was slightly higher in the preoperative group (10.7% versus 7.6%), this difference was not statistically significant. Marginally statistically significant treatment-by-age interactions appear to be emerging for survival and DFS, suggesting that younger patients may benefit from preoperative therapy, whereas the reverse may be true for older patients. [J Natl Cancer Inst Monogr 2001;30:96-102]

The rationale for testing preoperative (neoadjuvant) chemotherapy in the treatment of patients with operable breast cancer has evolved from preclinical (1,2) and clinical (3-9) observations as well as from hypothetical considerations of tumor cell kinetics (10,11). Nonrandomized studies (12-15) have demonstrated that preoperative chemotherapy administration results in substantial rates of clinical response but in generally low rates of pathologic complete response. By reducing primary tumor size, preoperative chemotherapy allowed some patients who otherwise would have required a mastectomy to undergo breast-conserving procedures. Since nonrandomized studies could not evaluate the relative efficacy of preoperative versus postoperative chemotherapy on overall survival (OS) and disease-free survival (DFS), several randomized trials (16-21) were implemented. Some of these trials (16,17), however, were not designed as direct comparisons of preoperative versus postopera-

tive chemotherapy and, therefore, could not provide a definitive answer to the pivotal question of whether OS and DFS can be improved by administering chemotherapy before, rather than after, surgery.

In 1988, the National Surgical Adjuvant Breast and Bowel Project (NSABP) initiated a randomized trial (B-18) to compare preoperative and postoperative chemotherapy in patients with operable breast cancer. The primary aim was to determine whether preoperative chemotherapy would result in improved OS and DFS relative to the same chemotherapy administered postoperatively. Secondary aims were to evaluate the response of the primary breast tumor and involved lymph nodes to preoperative chemotherapy, to correlate that response with outcome, and to determine whether preoperative chemotherapy would result in increased rates of breast-conserving surgery and decreased rates of ipsilateral breast tumor recurrence (IBTR). Findings with respect to local and regional response (20), 5-year outcome (21), compliance, and toxicity (21) have been published previously. This report updates the outcome results through 9 years of follow-up.

PATIENTS AND METHODS

Eligibility and Treatment Assignment

Eligibility criteria and treatment have been described previously (20,21). In summary, eligible patients had operable, palpable breast cancer (T1-3 N0-1 M0) diagnosed by fine-needle aspiration or core needle biopsy; open biopsy was not permitted. After stratification according to age (≤ 49 or ≥ 50 years of age), clinical tumor size (≤ 2.0 , 2.1-5.0, or > 5.0 cm), and clinical lymph node status (negative or positive), patients were randomly assigned to receive either surgery (lumpectomy and axillary lymph node dissection or modified radical mastectomy) followed by four cycles of doxorubicin (60 mg/m^2)/cyclophosphamide (600 mg/m^2) (AC) chemotherapy every 21 days or the same chemotherapy followed by surgery. Before randomization, surgeons were required to disclose the intended surgical procedure (lumpectomy or mastectomy) without considering the pos-

Affiliations of authors: N. Wolmark, National Surgical Adjuvant Breast and Bowel Project (NSABP), Pittsburgh, PA, and Department of Human Oncology, Allegheny General Hospital, Pittsburgh; J. Wang, NSABP Biostatistical Center and Department of Biostatistics, University of Pittsburgh; E. Mamounas, NSABP Breast Committee and Aultman Cancer Center, Canton, OH; J. Bryant, NSABP Biostatistical Center and Departments of Biostatistics and Statistics, University of Pittsburgh; B. Fisher, NSABP and Department of Surgery, University of Pittsburgh.

Correspondence to: Norman Wolmark, M.D., 320 E. North Ave., Pittsburgh, PA 15212 (e-mail: nwolmark@wpahs.org).

See "Note" following "References."

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sible downstaging effect of preoperative chemotherapy. Patients 50 years old or older received 10 mg tamoxifen orally twice a day for 5 years, starting after chemotherapy, regardless of hormone receptor status. Patients undergoing lumpectomies received breast irradiation, either after recovering from surgery (preoperative group) or after recovering from postoperative chemotherapy (postoperative group).

Accrual and Patient Characteristics

The study opened in October 1988 and closed in April 1993. Patient characteristics are summarized in Table 1. Of the 1523 patients, 763 were randomly assigned to the preoperative chemotherapy group and 760 to the postoperative chemotherapy group. Twenty-one patients were declared ineligible (seven postoperative and 14 preoperative; these totals include one patient in each group determined to have been ineligible subsequent to the first report of outcome) (21). Three of these patients had not given informed consent, six had advanced disease at the time of randomization, and three others were found to have had an open biopsy. The remaining nine cases were attributed to a variety of eligibility infractions.

Tumor Response

The primary tumor and axillary lymph nodes were clinically assessed before randomization. For patients receiving preoperative chemotherapy, breast tumor and lymph node measurements were also obtained both before each cycle of chemotherapy and before surgery. Preoperative patients were considered evaluable

for response if they had received at least two cycles of preoperative chemotherapy, had bidimensional tumor measurements recorded at the beginning of cycle 1, and had at least one additional set of tumor measurements recorded after cycle 2. The absence of clinical evidence of tumor in the breast by physical examination was categorized as clinical complete response (cCR). A clinical partial response (cPR) was assigned if the product of the two largest perpendicular diameters of the breast tumor had decreased by 50% or more. Progressive disease (cP) was assigned if there was a 50% or greater increase in tumor size. Patients whose breast tumor did not meet the criteria for cCR, cPR, or cP were considered to have stable disease (cS). After surgery, patients achieving a cCR were assessed further for evidence of pathologic response. Patients with cCR were classified as pathologic complete responders (pCR) if there was no histologic evidence of invasive carcinoma on pathologic examination of the surgical specimen and as pathologic nonresponders (pINV) otherwise. These findings were those reported by the institutional pathologists.

Outcome Measures

OS was defined as the time from study entry to death from any cause. DFS was defined as the time from randomization to local, regional, or distant treatment failure; occurrence of contralateral breast cancer; other second primary cancer; or death without evidence of breast or second primary cancer. Patients who became inoperable before surgery or in whom the tumor could not be completely resected were counted as local treatment failures. Recurrence-free survival (RFS) was defined as the time from randomization to local, regional, or distant treatment failure. In the calculation of RFS, occurrences of contralateral breast cancer, other second primary cancers, and deaths without evidence of recurrence were treated as censoring events.

Statistical Methodology

Treatment comparisons included in this report were based on the cohort of eligible patients with follow-up. Substantively identical findings were obtained when ineligible patients also were included in the analyses. Patients were analyzed according to their assigned treatment regardless of compliance or crossover. Survival curves were estimated using the Kaplan–Meier method, and treatment comparisons were made using the log-rank test stratified according to age, clinical lymph node status, and clinical tumor size as reported at randomization. The Cox proportional hazards model was used to compute relative risks (RRs) and 95% confidence intervals (CIs), to examine the effect of prognostic variables, and to test for interactions between treatment and covariates. Treatment comparison of rates of IBTR was based on the occurrence of IBTRs as first events. The Mantel–Haenszel approach was used to control for patient age and clinical tumor size and was based on the Poisson occurrences model.

In preoperative patients, correlation between primary tumor response and subsequent outcome is clinically relevant primarily because it might enable one to distinguish patients who, after surgery, had an excellent prognosis from those whose prognosis was poor and who, therefore, might be candidates for additional therapy. For this reason, in correlation analyses, the outcome variables OS, DFS, and RFS were measured from the date of the surgery to the time of the event, and the analyses were restricted to eligible preoperative patients who were evaluable for response, had undergone surgery, and were clinically free of dis-

Table 1. Patient eligibility, follow-up, and entry characteristics

Eligibility, follow-up, and entry characteristics	Treatment group*		
	Postoperative AC	Preoperative AC	Total
Eligibility			
Randomized	763	760	1523
Ineligible	7	14	21
Eligible without follow-up	5	4	9
Analyzed	751	742	1493
Follow-up of analyzed patients			
Mean time on study, y	9.5	9.5	9.5
Characteristics of analyzed patients			
Age, %			
≤49 y	52	51	52
50–59 y	26	25	26
≥60 y	22	23	23
Menopausal status, %			
Premenopausal or perimenopausal	51	49	50
Postmenopausal	48	50	49
Unknown	1	1	1
Race, %			
White	81	81	81
Black	11	9	10
Other	7	8	7
Unknown	1	2	1
Clinical tumor size, %			
≤2.0 cm	27	29	28
2.1–5.0 cm	60	58	59
≥5.1 cm	13	13	13
Mean tumor size ± standard deviation	3.5 ± 1.8	3.5 ± 1.8	3.5 ± 1.8
Clinical lymph node status, %			
Negative	74	74	74
Positive	26	26	26

*AC = doxorubicin + cyclophosphamide.

ease as of the date of surgery. Of 682 such patients, 247 (36%) had primary tumor responses that were classified as cCR, 295 (43%) were cPR, 118 (17%) were cS, and 22 (3%) were cP. Because few patients experienced cP, the cS and cP categories were combined in these analyses. Patients in the combined category are referred to as clinical nonresponders (cNR). Statistical tests of association between clinical tumor response and outcome variables assumed an ordinal relationship between response categories. The tests were obtained by computing a response score for each patient (1 = cCR, 2 = cPR, and 3 = cNR) and introducing this score as a covariate in Cox proportional hazards models.

Of the 247 patients with complete clinical responses, 88 (13% of 682) were further classified in terms of pathologic response as pCR, and 159 (23% of 682) were pINV. Tests for association between overall primary tumor response and outcome variables were obtained by assigning an ordinal response score to each patient (1 = pCR, 2 = pINV, 3 = cPR, and 4 = cNR) and introducing this score into a proportional hazards model. Tests for association were carried out both ignoring and controlling for other prognostic variables.

Results presented here are based on data received at the NSABP Biostatistical Center as of June 30, 2000. The mean time on study is 9.5 years. All *P* values are two-sided.

RESULTS

Survival

There have been 218 deaths in the postoperative group and 221 in the preoperative group. There continues to be no statistically significant difference in survival between the two groups ($P = .80$; RR = 1.02; 95% CI = 0.84 to 1.21). The 5-year survival was 81% in the postoperative group and 80% in the preoperative group. The 9-year survival was 70% in postoperative patients and 69% in preoperative patients (Fig. 1).

Disease-Free Survival

There have been 338 events in the postoperative group and 323 in the preoperative group. There was no difference in DFS between the two groups ($P = .50$; RR = 0.95; 95% CI = 0.88 to 1.10). The 5-year DFS was 67% for both treatment groups. The 9-year DFS was 53% in the postoperative group and 55% in the preoperative group (Fig. 1).

First Reported Sites of Treatment Failure

As has been reported through 5 years of follow-up, there continue to be no statistically significant differences in the rates

of treatment failure at any specific site (Table 2). Although there was a trend toward a higher rate of IBTR with preoperative chemotherapy, this difference was not statistically significant ($P = .12$): There were 34 (7.6%) IBTRs among 448 patients who underwent lumpectomy in the postoperative group and 54 (10.7%) among 503 such patients in the preoperative group. There was a strong correlation between age and rate of IBTR ($P = .00003$), with higher IBTR rates in women less than 50 years of age (13.1%) when compared with the rates of those 50 years of age or over (5.2%) (Table 3). Of note is the fact that women 50 years of age or older at randomization received tamoxifen, whereas those under 50 years of age did not. Clinical tumor size did not appear to correlate with the rate of IBTR ($P = .59$; Table 3). Although patients with a complete pathologic response (pCR) appeared to have a somewhat lower rate of IBTR than the remaining patients, the association between primary tumor response and IBTR rate was not statistically significant ($P = .12$; Table 3).

A marginally statistically significant increase ($P = .04$) was reported initially in the rate of IBTR found in patients who were converted from proposed mastectomy to lumpectomy after preoperative chemotherapy when compared with those patients who had a lumpectomy as initially planned before randomization (21). This trend persists through 9 years of follow-up. The rate of IBTR is 11/69 (15.9%) in preoperative patients downstaged to lumpectomies, as compared with 43/434 (9.9%) in preoperative patients who received lumpectomies as originally planned. The difference, however, is explained partially by corresponding differences between the age distribution of downstaged patients to that of patients having lumpectomies as planned and is no longer statistically significant after controlling for patient age and initial clinical tumor sizes ($P = .14$).

Subset Analyses

There was no evidence for treatment-by-covariate interaction for either clinical lymph node status or clinical tumor size. Treatment-by-age interaction, however, was marginally statistically significant for both OS and DFS ($P = .04$ for OS; $P = .053$ for DFS). In women 49 years old or younger, there appeared to be an advantage for preoperative chemotherapy; at 9 years of follow-up, OS was 71% versus 65% and DFS was 55% versus 46% in favor of patients treated with preoperative chemotherapy. Conversely, in women 50 years old or older, there seemed to be an advantage in favor of postoperative chemotherapy; at 9 years of follow-up, OS was 75% versus 67% and DFS was 60% versus

Fig. 1. Overall survival and disease-free survival according to treatment through 9 years of follow-up (Postop = postoperative chemotherapy; Preop = preoperative chemotherapy).

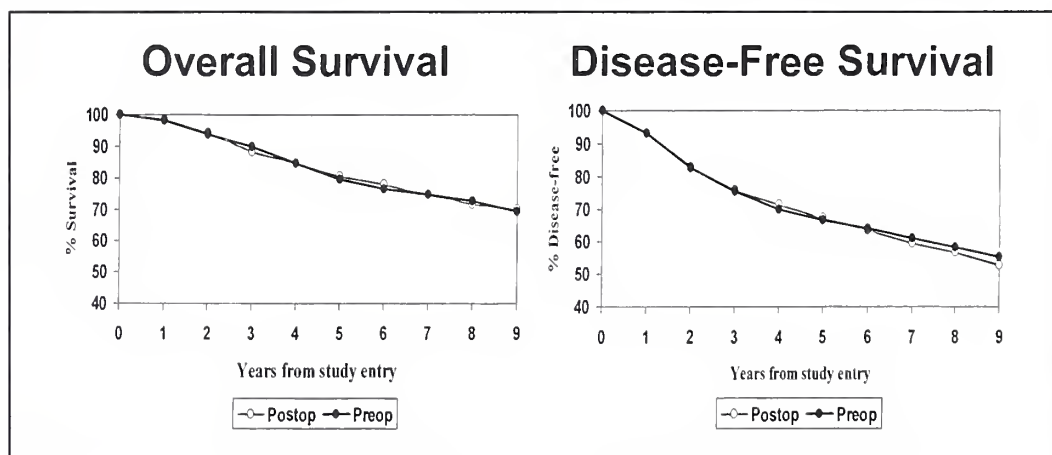


Table 2. First reported sites of treatment failure

Type and site of failure	Treatment group			
	Postoperative AC		Preoperative AC	
	No.	%	No.	%
Clinically inoperable	0	0	1	0.1
Gross residual disease	11	1.5	8	1.1
IBTR only*	34	7.6	54	10.7
Other local recurrence	21	2.8	21	2.8
Regional recurrence	30	4.0	24	3.2
Distant metastasis (except opposite breast)	155	20.6	145	19.5
Second primary cancer (except opposite breast)	32	4.3	29	3.9
Opposite breast cancer	30	4.0	25	3.4
Dead, no evidence of disease	25	3.3	16	2.2
Total first events	338	45.0	323	43.5
Alive, event free	413	55.0	419	56.5
Total No. of patients	751	100	742	100

*Percentages for ipsilateral breast tumor recurrence (IBTR) are based on the numbers of patients who received lumpectomies.

Table 3. Clinical factors associated with ipsilateral breast tumor recurrence (IBTR)

Clinical factor	Treatment group		
	Postoperative AC, % of patients with IBTR	Preoperative AC, % of patients with IBTR	Total, % of patients with IBTR
Age, y			
≤49	10.7	15.2	13.1
≥50	4.2	6.1	5.2
Clinical tumor size			
<3 cm	6.6	11.6	9.3
≥3 cm	8.3	10.1	9.3
Clinical and pathologic tumor response			
cCR*	N/A	9.8	
pCR	N/A	6.7	
pINV	N/A	11.5	
cPR	N/A	11.8	
cNR (cSD and cPD)	N/A	13.0	
Procedure after preoperative chemotherapy			
Lumpectomy vs. planned mastectomy	N/A	15.9	
Lumpectomy as planned	N/A	9.9	

*cCR = clinical complete response; pCR = pathologic complete response; pINV = pathologic nonresponders; cPR = clinical partial response; cNR = clinical nonresponders; cSD = clinical stable disease; cPD = clinical progressive disease; N/A = not applicable.

56% in favor of postoperative chemotherapy. Within either age group, however, the preoperative versus postoperative treatment comparison did not achieve statistical significance for either OS or DSF (in younger women, RR = 0.85 and $P = .22$ for OS and RR = 0.85 and $P = .11$ for DFS; in older women, RR = 1.28 and $P = .08$ for OS and RR = 1.09 and $P = .44$ for DFS).

Association Between Clinical Response and Outcome

Patients in the preoperative chemotherapy group were categorized according to clinical response (cCR, cPR, or cNR). Through 9 years of follow-up, there continues to be an apparent

association between clinical response and outcome. This association now has become statistically significant not only for DFS and RFS (as was the case through 5 years of follow-up) but also for OS (OS: $P = .005$; DFS: $P = .0008$; RFS: $P = .0002$). OS at 9 years was 78% in patients with cCR, 67% in patients with cPR, and 65% in those with cNR. The rates of DFS were 64%, 54%, and 46%, respectively. The statistically significant association between clinical response and outcome persisted after adjustment for clinical tumor size at randomization, clinical lymph node status, and age at randomization (OS: $P = .04$; DFS: $P = .004$; RFS: $P = .0008$).

Association Between Pathologic Response and Outcome

Similar to the results through 5 years of follow-up, the outcome for patients who achieved a pCR continues to be superior to that of those with a cCR with residual invasive cancer on pathologic examination (pINV) or to those patients failing to achieve a cCR (Fig. 2). At 9 years, the OS rate for patients achieving a pCR was 85% as compared with 73% for patients with pINV. For DFS, the respective rates were 75% and 58%. Overall primary tumor response graded as pCR, pINV, cPR, or cNR was strongly associated with all outcome measures (OS: $P = .0008$; DFS: $P = .00005$; RFS: $P = .0002$). These associations persisted after adjustment for clinical tumor size at randomization, clinical lymph node status, and age at randomization (OS: $P = .006$; DFS: $P = .0004$; RFS: $P = .00006$). After adjustment for the other prognostic variables, patients with pCR had a 50% reduction in the risk of death when compared with the group as a whole (RR = 0.50; 95% CI = 0.32 to 0.78), those with pINV had an 8% increase (RR = 1.08; 95% CI = 0.81 to 1.42), those with cPR had a 28% increase (RR = 1.28; 95% CI = 1.01 to 1.62), and those with cNR had a 45% increase (RR = 1.45; 95% CI = 1.11 to 1.90).

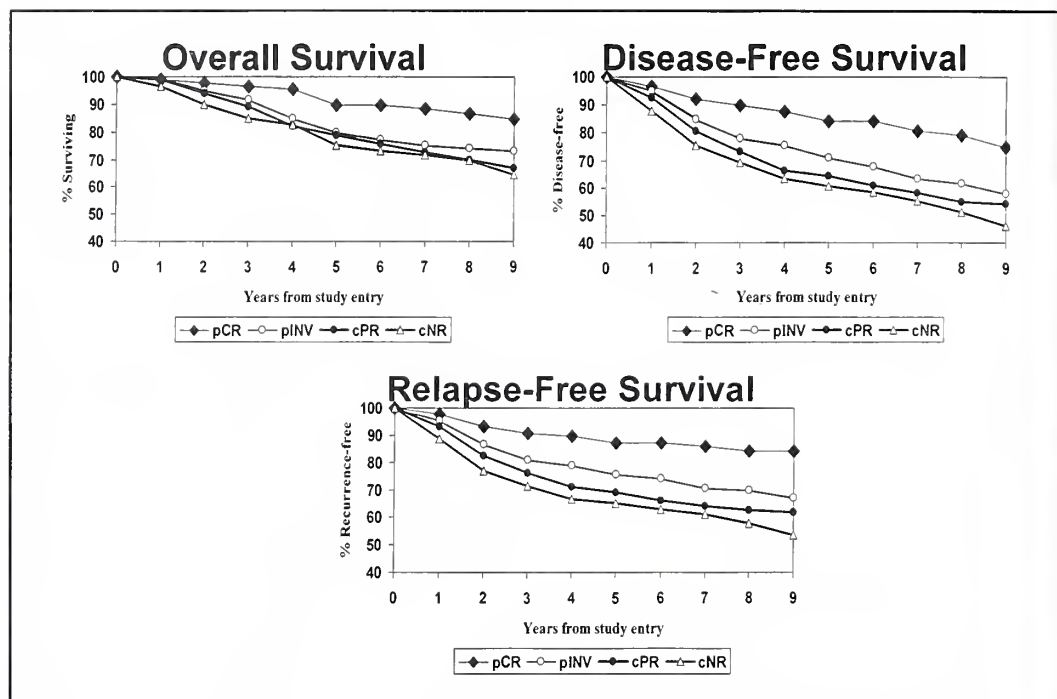
Prognostic Importance of Primary Tumor Response After Controlling for Pathologic Lymph Node Status

As expected, pathologic lymph node status was a strong predictor of outcome in both preoperative and postoperative patients ($P < .0001$ for OS, DFS, and RFS in either cohort). In patients treated with preoperative chemotherapy, the resulting pathologic lymph node status was also, not surprisingly, related to primary tumor response. The Spearman correlation between the number of involved lymph nodes and primary tumor response (pCR, pINV, cPR, or cNR) was 0.22 ($P < .0001$). To provide a formal test of the hypothesis that primary tumor response contributes prognostic information beyond that provided by pathologic lymph node status, proportional hazards models were fitted to the preoperative cohort, including a score variable representing primary tumor response after stratifying for pathologic lymph node status (0, 1–3, or ≥4). Results demonstrated that primary tumor response does contribute additional prognostic information over and above pathologic lymph node status (OS: $P = .06$; DFS: $P = .006$; RFS: $P = .004$). Conversely, pathologic lymph node status was also strongly prognostic even after controlling for primary tumor response ($P < .0001$ for OS, DFS, and RFS).

DISCUSSION

The mature results of the B-18 trial presented here continue to support the conclusions of previous reports (21). They demonstrate that, through 9 years of follow-up, the outcome for patients

Fig. 2. Comparison of outcome of patients treated with preoperative chemotherapy according to primary breast tumor response.



treated with preoperative chemotherapy is similar to the outcome for those treated with standard adjuvant chemotherapy. These results do not support the Goldie–Coldman hypothesis, which proposes that, as a tumor cell population increases, an ever-expanding number of drug-resistant phenotypic variants arises that are more difficult to eradicate with chemotherapy.

Two smaller European trials (16,17) that compared preoperative with postoperative chemotherapy had outcome results discordant with those of B-18. These trials demonstrated a survival advantage for preoperative chemotherapy with no differences in DFS. In both trial designs, however, there were imbalances in the systemic and local therapy administered to the two groups. Although all patients in the preoperative chemotherapy group received chemotherapy, only lymph node-positive patients did so in the postoperative chemotherapy group. Similarly, more patients received surgery in the postoperative chemotherapy group than in the preoperative chemotherapy group, with a resulting increase in the rate of local recurrence in the latter. The outcome results of another trial (19), conducted at the Royal Marsden Hospital in England, were similar to our results. In that trial, a total of 309 patients were randomly assigned either to receive four preoperative cycles of chemoendocrine therapy followed by four postoperative cycles of the same therapy or to receive all eight cycles of therapy postoperatively. At a median follow-up of 48 months, there were no statistically significant differences between the two groups in terms of local relapse, metastatic relapse, or OS.

Study B-18 continues to demonstrate a statistically significant association between clinical/pathologic tumor response to preoperative chemotherapy and long-term outcome. This association does not support the Skipper concept, in which the response of a primary tumor to chemotherapy may not necessarily reflect the response of micrometastatic disease. Furthermore, it suggests that the underlying biologic factors required for pathologic complete response may also confer true chemosensitivity to micrometastatic disease, allowing long-term improvement in outcome as opposed to a temporary delay in recurrence. This is in

contrast to the metastatic disease setting, where cCR generally results in only temporary prolongation in time to progression.

As reported previously (20), administration of preoperative chemotherapy resulted in statistically significantly more lumpectomies, particularly among patients with tumors greater than 5 cm in diameter at randomization. This was accompanied by a statistically nonsignificant increase in the rate of IBTR (10.7% in the preoperative chemotherapy group versus 7.6% in the postoperative chemotherapy group). This can be attributed partially to the fact that the former group contained some downstaged patients who may have been at higher risk for local recurrence irrespective of the assigned treatment arm. Although, in the present report, IBTR rates were not statistically significantly associated with the initial clinical tumor size, the rates were somewhat higher in patients for whom a mastectomy was planned at the time of randomization but for whom a lumpectomy was performed after preoperative chemotherapy (15.9%), as opposed to those for whom a lumpectomy was planned from the beginning (9.9%).

The noted increase in IBTR rates in patients under 50 years of age when compared with those in patients 50 years of age or older is not surprising. Younger patients generally have more aggressive disease than older patients; this results in a higher rate of local and systemic recurrence. But even so, the largest part of the difference probably is caused by the inherent design of the study, whereby tamoxifen was administered only to patients 50 years old or older at randomization, irrespective of estrogen receptor status. Randomized trials have shown convincingly that tamoxifen markedly reduces the rate of IBTR after lumpectomy and breast radiotherapy in both older and younger women (22).

The observed marginally statistically significant interaction between treatment effect and age at randomization is enigmatic. In the B-18 data, patients under 50 years of age appeared to show a greater benefit from preoperative chemotherapy than from postoperative chemotherapy. In contrast, patients 50 years old or older appeared to benefit more from postoperative chemotherapy. The most likely explanation for these results is that

they occurred by chance alone and that a true interaction between treatment and age does not exist. Alternatively, the overview analyses of the Early Breast Cancer Trialists' Collaborative Group (3) indicate that the effects of chemotherapy are most apparent in younger women, so it is not inconceivable that the benefit of preoperative chemotherapy relative to postoperative treatment could be age dependent as well. To the extent that younger patients present more often than older patients with estrogen receptor-negative tumors, this conjecture is consistent with a recent International Breast Cancer Study Group retrospective analysis (23) suggesting that there may be a preferential benefit to early initiation of adjuvant chemotherapy in premenopausal patients whose tumors do not express the estrogen receptor. Because of limitations on the assay of hormone receptors in the early years of the B-18 study, data are not available to address this issue. In any case, although intriguing explanations and hypotheses may be invoked, until additional data are forthcoming, the interpretation of the findings remains speculative.

On the basis of both the results presented here and those reported previously (20,21), preoperative chemotherapy can be used instead of postoperative adjuvant chemotherapy, and its use would be most appropriate for patients who wish to preserve their breasts but who have tumors too large for breast-conserving surgery. Another potential advantage of preoperative chemotherapy is the resulting classification of patients in different categories of clinical and pathologic tumor response, which can be used as a prognostic factor for outcome and as a guideline for further locoregional and systemic therapy (24).

The development of taxanes and the demonstration of their marked antitumor activity in patients with advanced breast cancer provided the opportunity to examine further some of the concepts that have emerged from the B-18 trial. The NSABP recently completed accrual to Protocol B-27, a randomized trial designed to determine whether the preoperative or postoperative administration of docetaxel after preoperative AC therapy will prolong OS and DFS rates when compared with four courses of preoperative AC therapy alone (25,26). Equally important are the secondary aims of this trial, which are to determine whether the administration of preoperative docetaxel after preoperative AC therapy will further increase the clinical and pathologic response rates of primary breast tumors, whether it will result in further axillary lymph node downstaging, and whether it will increase the use of lumpectomy. A comparison of the group receiving postoperative docetaxel after preoperative AC therapy with the group receiving preoperative AC alone will identify whether any improvement in outcome will be evident in subgroups of patients, i.e., in patients with residual positive lymph nodes after preoperative AC. Two ancillary studies to the B-27 trial evaluate serum and tumor biomarkers as they relate to outcome and response to preoperative AC and/or docetaxel chemotherapy. Thus, it will be possible, using the collected materials, to evaluate the prognostic and predictive value of a panel of biomarkers, including HER2, p53, p-glycoprotein, bcl-2 Ki67, and array-based comparative genomic hybridization.

Perhaps the greatest potential of preoperative adjuvant therapy is yet to be realized. This is a unique setting in which the tumor is readily accessible while the patient is undergoing treatment. Thus, a potentially powerful tool could become available whereby molecularly characterized tumor discriminants could be correlated with the efficacy of preoperative adjuvant treatment and, more importantly, with subsequent survival. Although

it is premature to suggest that objective tumor regression during the course of adjuvant therapy is a definitive surrogate marker for eventual patient outcome, the data for NSABP Protocol B-18 suggest that this is a distinct possibility.

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Who Should Not Receive Adjuvant Chemotherapy?

International Databases

Jonas Bergh, Marit Holmquist

The optimal selection of patients for adjuvant therapy, avoiding overtreatment and undertreatment, of disease is a significant challenge in the management of early breast cancers. Population-based cohorts in Denmark and in two Swedish health care regions were investigated to identify patients with breast cancer who have a sufficiently low risk of recurrence without adjuvant therapy. Published data on different calcification patterns were also included from the randomized Swedish mammography two-county study. The Danish Breast Cancer Group's population-based registry revealed that patients with lymph node-negative and estrogen receptor- or progesterone receptor-positive cancers of histological grade I that were less than 20 mm in size had a 5-year survival rate similar to age-matched groups without breast cancer. Data from the Stockholm Breast Cancer Group identified a similar risk group (no information on cancer grade) with an approximate 10% risk of dying from breast cancer after 10 years without any adjuvant therapy. In women older than 50 years, approximately 20% died of other causes. Mammographically and lymph node-negative-detected cancers that are less than 15 mm in size generally have an excellent survival outcome, excluding patients with casting calcifications. Patients who have lymph node-negative breast cancers that are less than 20 mm in size, combined with estrogen receptor positivity, can be identified as a low-risk group. The vast majority of these patients are unlikely to benefit from the addition of conventional chemotherapy, but some of them may. The dilemma is that we cannot identify these patients prospectively because of the lack of relevant predictive factors for chemotherapy. [J Natl Cancer Inst Monogr 2001;30:103-8]

Systemic therapy with tamoxifen therapy and chemotherapy are the most important adjuvant modalities for improving the survival of women with primary breast cancer (1-3). A critical issue, however, is the identification of those patients who should be offered adjuvant chemotherapy based on a sufficiently high risk for recurrence. Scandinavian databases will be used in this article to identify breast cancer patients who potentially will be cured by only local or locoregional therapies, which makes the use of adjuvant therapy, especially chemotherapy, less necessary.

SELECTION OF PATIENTS FOR ADJUVANT THERAPY—POSSIBILITIES AND SHORTCOMINGS

Despite the stepwise improvements offered by the combination of different adjuvant therapies, major problems still exist regarding the selection of patients to whom such treatment should be offered. The critical problem is finding the optimal balance between overtreatment and undertreatment. One may

accept overtreatment of relatively large patient groups if the long-term side effects are minimal. This seems to be the case for many of our standard chemotherapy regimens (2). Furthermore, economic cost-benefit analyses for standard adjuvant chemotherapy appear acceptable; the cost per year of life saved is lower than some other accepted medical interventions, especially when compared with other nonmedical procedures (4,5). The cutoff levels and recommendations for adjuvant therapy vary between countries and regions in the world, most likely reflecting differing medical, cultural, and economic assessments.

The lack of established predictive factors for chemotherapy is a major obstacle to improving outcomes. Many patients will relapse despite having received adjuvant chemo-endocrine therapy. Theoretically, relapse may not have occurred if alternate combinations of drugs, based on analyses of relevant predictive factors, were administered "up front." In the future, a detailed molecular "fingerprint" of each cancer could help to guide a tailored approach for each patient, together with an appreciation of individual pharmacokinetic variations (6-8). Recent data with the use of microarray technology demonstrate a very high degree of complexity (9). This should stimulate further research aimed at tailored therapy approaches.

International Databases

Information on the effects of adjuvant chemotherapy in breast cancer patients has traditionally been obtained from randomized studies. How representative the patients in randomized studies are compared with the general population, however, is less well-known. The selection bias of some clinical trials is most likely quite marked. A complementary approach to obtaining therapy and outcome data would be to study different population-based cancer and therapy registries, which are available in some parts of Europe. This review will discuss these registry results in some detail, with the aim of identifying patients unlikely to benefit from adjuvant chemotherapy.

Danish Breast Cancer Group (DBCG)

Established in 1977, the DBCG has carried out multiple national adjuvant and randomized studies. In parallel, they have created a registry of "all" new breast cancers occurring in Denmark since 1977. The registry contains some 60 000 reports. On

Affiliation of authors: Jonas Bergh, Radiumhemmet, Karolinska Institute and Hospital, Stockholm, Sweden, Marit Holmquist, Regional Oncological Centre, Uppsala, Sweden.

Correspondence: Jonas Bergh, M.D., Ph.D., Department of Oncology, Radiumhemmet, Karolinska Hospital and Institute, S-171 76 Stockholm, Sweden (e-mail: jonas.bergh@cck.ki.se).

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the basis of 30 000 of the reported breast cancers, a low-risk group has been identified. Patients in the DBCG low-risk group had the following characteristics: having primary tumor size of less than or equal to 20 mm, having negative axillary lymph nodes, being Bloom–Richardson grade 1, and being estrogen or progesterone receptor positive (≥ 10 fmol/mg protein or $\geq 10\%$ positive cells with the use of immunohistochemistry).

No adjuvant systemic therapy, tamoxifen therapy, or chemotherapy was given to this group. Radiotherapy was given to the scar area if the mastectomy operation was not radical, and it was also given to the remaining breast parenchyma in patients treated with breast-conserving surgery. The 5-year survival rate was calculated for this group. The premenopausal cohort had a 98% 5-year survival rate that was identical to that of a matched control group without breast cancer. The postmenopausal cohort had a 91% 5-year survival rate compared with a 92% 5-year survival rate for the control group (10) (Mouridsen H: personal communication) (Table 1). On the basis of these figures, the addition of adjuvant therapy, especially chemotherapy, should be questioned. However, for this low-risk group, a 5-year follow-up period is too brief, as will be discussed.

Regional Swedish Breast Cancer Registries

Sweden has six health care regions with regional breast cancer registries that maintain information on primary tumor status as well as on primary therapy. Data have been obtained for this article from the two largest regions—Stockholm-Gotland and Uppsala-Örebro.

Stockholm Breast Cancer Group

The Stockholm Breast Cancer Group, Sweden, was established in 1976. Their database contains information on approximately 20 000 breast cancer patients (Rutqvist LE: personal communication). The population base is 1.9 million inhabitants. The registry has high coverage and follow-up of the breast cancers within the defined geographic region. A low-risk group was identified from a total of 15 842 patients undergoing primary surgery for breast cancer during the period from 1976 through 1996. The patients' status was followed up for events until December 31, 1997, and the data were matched against the nationwide death cause registry. The low-risk group from the Stockholm Breast Cancer Group had the following characteristics: having primary tumor size of less than 20 mm, having negative axillary lymph nodes, and being estrogen receptor positive (≥ 0.05 fmol/ μ g DNA).

A total of 1929 (12.2%) patients who had not received adjuvant systemic therapy were identified from the registry, which held information on 15 842 patients during the time period 1976 through 1996. The median follow-up period was 11.9 years for the 1929 patients, 500 of whom were younger than 50 years.

The overall survival rate at 10 years for the "postmenopausal" group was 70%, and the corresponding figure for those younger than 50 years was 89% (Fig. 1, a and b, Table 1). The cumulative incidence of breast cancer deaths was approximately 10% for both age strata (Table 1). For the younger age group, most breast cancer deaths were recorded after 4 years of follow-up (Fig. 1, a). For women aged 50 years and older, non-breast cancer and breast cancer deaths increased linearly during the entire 10-year

Table 1. The three population-based low-risk cohorts

Study group	Size of the registry	Size of the studied group	Low-risk criteria	Adjuvant therapy given	Outcome—survival rate (age, y)
Danish Breast Cancer Group	Approximately 60 000	670 (premenopausal) 1800 (postmenopausal)	Tumor < 20 mm Lymph node negative Grade 1 Estrogen receptor or progesterone receptor positive	No	5-y survival rate 98% survival rate premenopausal 91% survival rate postmenopausal
Stockholm Breast Cancer Group	15 842, study period 1976–1996	500 < 50 y 1429 \geq 50 y	Tumor < 20 mm Lymph node negative Estrogen receptor positive	No	5-y survival rate 97% overall survival rate (<50) 97% breast cancer survival rate (<50) 89% overall survival rate (\geq 50) 97% breast cancer survival rate (\geq 50) 10-y survival rate 89% overall survival rate (<50) 90% breast cancer survival rate (<50) 70% overall survival rate (\geq 50) 92% breast cancer survival rate (\geq 50)
Uppsala-Örebro Breast Cancer Group	8232, study period 1993–1998	735	Tumor < 10 mm Lymph node negative	No	5-y survival rate 95% overall survival rate 100% relative survival rate 8-y survival rate 87% overall survival rate 97% relative survival rate
		523	Tumor 10–20 mm Lymph node negative Estrogen receptor positive Low S phase	No	5-y survival rate 93% overall survival rate 100% relative survival rate 8-y survival rate 83% overall survival rate 95% relative survival rate
		94	Tumor 10–20 mm Lymph node negative Estrogen receptor negative High S phase	No	5-y survival rate 87% overall survival rate 94% relative survival rate 8-y survival rate 77% overall survival rate 88% relative survival rate

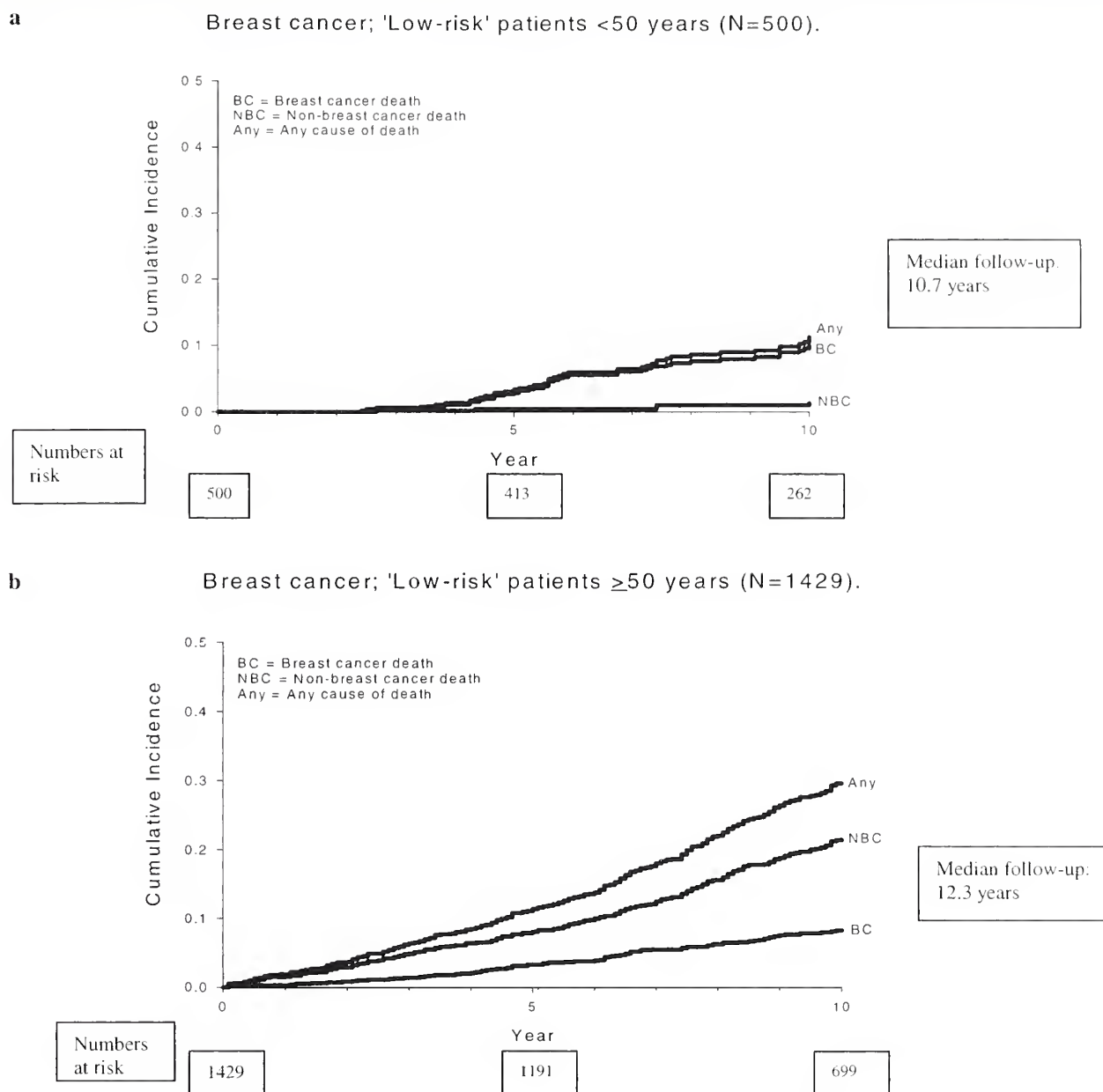


Fig. 1. The low-risk group was identified by lymph node negativity, a tumor size of less than 20 mm, and an estrogen receptor status of 0.05 fmol/ μ g DNA or greater. None of the patients had received adjuvant systemic therapy (chemotherapy or tamoxifen therapy).

period. Approximately two thirds of deaths were non-breast cancer related (Fig. 1, b). The nearly 10% risk of dying of breast cancer for both age strata at 10 years would argue for the use of adjuvant hormonal therapy in estrogen receptor-positive tumors. Adjuvant tamoxifen therapy for 5 years will give a 47% proportional reduction of recurrence and should therefore be considered (1).

Uppsala-Örebro Breast Cancer Group

The regional breast cancer registry for the Uppsala-Örebro region was activated on September 1, 1992, by the Uppsala-

Örebro Breast Cancer Study Group. The population base is approximately 1.9 million inhabitants. The registry did not initially contain information on preoperative therapy. For this report, only lymph node-negative patients treated during the time period from 1993 through 1998 were included. Of these, 3029 had not received postoperative adjuvant chemotherapy or tamoxifen therapy. The outcome for those patients was compared with mortality rates for age-matched groups. The relative survival rate is defined as the observed survival in the patient group divided by the expected survival rate of a comparable group in the general population followed during the same calendar years (11). All survival rates are presented with 95% confidence intervals (CIs).

Patients with a tumor size of less than 10 mm demonstrated an overall survival rate at 8 years of 87% (95% CI = 0.82 to 0.91) and a relative survival rate of 97% (95% CI = 0.92 to 1.01) based on 735 patients (Table 1). Nine hundred and seventy-seven patients with primary tumor sizes of 10–14 mm had an overall survival rate at 8 years of 85% (95% CI = 0.80 to 0.88) and a relative survival rate of 96% (95% CI = 0.91 to 1.0). For the 897 patients who had tumors sized 15–20 mm, the overall survival rate was 81% (95% CI = 0.75 to 0.85) at 8 years and the relative survival rate was 94% (95% CI = 0.88 to 0.99).

Five hundred twenty-three patients with a primary breast cancer sized 10–20 mm with positive estrogen receptors and low S

phase were compared with 94 patients with a primary breast cancer with high S phase and negative estrogen receptors (Table 1). The 8-year overall survival rate for the first group was approximately 83% (95% CI = 0.75 to 0.89), for the latter group, it was 77% (95% CI = 0.63 to 0.87) (Fig. 2, a) ($P = .048$; Pearson log-rank test). The corresponding relative survival figures are depicted in a separate figure (Fig. 2, b), 95% for the low-risk group (95% CI = 0.86 to 1.01), and 88% for the high-risk group (95% CI = 0.72 to 1.0) ($P = .052$; Pearson log-rank test).

On the basis of the study of 1021 lymph node-negative patients with estrogen receptor-positive breast cancer 10–20 mm in size, the overall survival rate at 8 years was 84%. The corre-

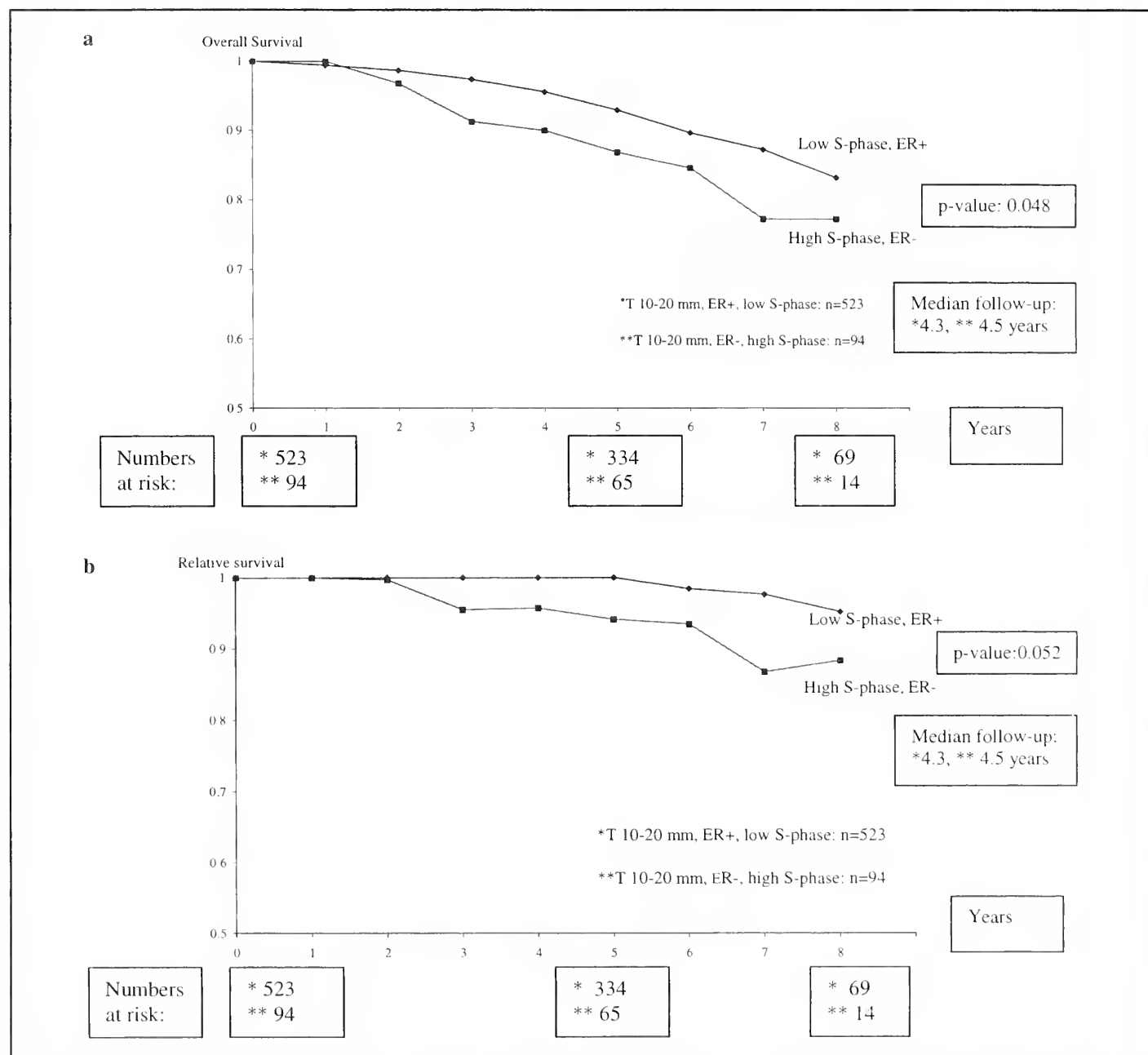


Fig. 2. Data from the Uppsala-Örebro region population-based cancer registry, tumors diagnosed from 1993 through 1998, axillary lymph node negative breast cancers with estrogen receptors (ER) and low S phase or estrogen receptor negativity with high S phase, and none of the patients received postoperative therapy with chemotherapy or tamoxifen therapy.

sponding value for estrogen receptor-negative cancers was 74%, based on the study of 291 patients. Indirect comparisons thus indicate that S phase added no prognostic information.

Identification of Low Risk Groups with the Use of Mammographic Patterns

From July 1977 through March 1986, a population-based and randomized mammography screening program was held in two Swedish counties. The study demonstrated a reduced breast cancer mortality rate for the screened group (12). The study has recently been updated with information on 2468 breast cancers (13). The authors conclude that mammography screening and histopathologic demonstration of axillary lymph node-negative breast cancers will enable the identification of a low-risk subgroup, which they believe should be recommended for less radical treatment. On the basis of these findings, the authors further investigated 343 primary breast cancers with a primary tumor size ranging from 1 to 14 mm (14). The long-term survival rate was determined to be 95% for the group of patients with a primary tumor size ranging from 1 to 9 mm without casting calcifications (14). However, 19 (14%) of 138 patients with tumors 1–9 mm in size had a specific type of calcification described as “casting” calcifications. These patients accounted for 73% of the breast cancer deaths ($P<.001$) in this analysis (14).

DISCUSSION

Adjuvant hormonal therapy and chemotherapy alone and in combination have been instrumental in reducing breast cancer mortality rates by 25%–30% in the U.K. and the United States since 1970 (15). This positive development has been recorded during the last decade. This major improvement is likely explained by the use of adjuvant therapies, stage migration resulting from increased awareness, and screening mammography. The important message is that breast cancer mortality can be substantially reduced by different strategies. The use of adjuvant therapy has until now been restricted to lymph node-positive and lymph node-negative breast cancers with high-risk features. The potentially wider use of adjuvant therapy on larger patient groups may result in further absolute survival improvements, but the benefit–risk equation for the low-risk groups will be less favorable.

Patients with small breast cancers (<10–15 mm) without axillary lymph node metastases, combined with favorable biologic markers (histologic grade I, receptor positive, and low S phase), are likely to experience only a small additive effect of adjuvant chemotherapy in combination with tamoxifen therapy, yet this statement is challenged by the recent retrospective analysis of five National Surgical Adjuvant Breast and Bowel Project studies (16). This analysis of lymph node-negative breast cancer trials demonstrates a statistically significant survival improvement when chemotherapy is added to tamoxifen therapy and surgery, even in patients with estrogen receptor-positive cancers less than 1 cm in diameter (16). The overall survival rate after 8 years for the groups studied by the National Surgical Adjuvant Breast and Bowel Project who were treated with surgery alone is on the same order or slightly better than the population-based figure from the Uppsala–Örebro region.

The data from the two Swedish regions demonstrate that the

mortality for the lymph node-negative group and the estrogen receptor-positive groups will occur only after several years of follow-up. These data argue strongly that 5 years of follow-up is too short, especially for the younger age group. Furthermore, the routine follow-up should be different for the low-risk groups with late-occurring relapses compared with patients at higher risk who usually relapse earlier.

The retrospective analysis of the Swedish two-county study indicates that different mammographic patterns may be used for selecting patients at very low risk versus those with a markedly higher risk, despite identical tumor size (14). Whether the calcification patterns can be generally applied and whether the selected high-risk patients will benefit from adjuvant therapy have yet to be demonstrated. The risk–benefit effects in medical and economic terms for treating low-risk patients with adjuvant chemotherapy have not been sufficiently investigated. Further studies are warranted.

CONCLUSION

In conclusion, breast cancer demonstrates a wide span in malignancy potential and response to therapy, and adjuvant chemotherapy cannot be recommended for all patients with breast cancer. Those patients with lymph node-negative, estrogen receptor-positive small tumors should consider carefully the risk and benefits involved.

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NOTES

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Who Should Not Receive Chemotherapy? Data From American Databases and Trials

Monica Morrow, Helen Krontiras

The demonstration of the effectiveness of chemotherapy in both premenopausal and postmenopausal women, regardless of estrogen receptor (ER) status, raises the question of whether all breast cancer patients should receive chemotherapy. Several patient groups with such a favorable long-term prognosis that they will obtain an extremely small benefit from chemotherapy can be identified. They include patients with lymph node-negative tumors of 1 cm or less in size, those with grade 1 tumors between 1.1 and 2.0 cm in size, and those with tumors of favorable histologic type (tubular and mucinous) up to 3 cm in size. A patient subgroup in which it is not clear that the benefits of chemotherapy routinely exceed the risks is postmenopausal women with ER-positive, lymph node-negative cancers receiving tamoxifen. There is a wide variation in prognosis in this group, and chemotherapy should be reserved for those at high risk of recurrence. Finally, no benefit for chemotherapy in women aged 70 years and older has been identified. The high rate of death from causes other than breast cancer may negate small survival benefits, and after adjustment for quality of life, the duration of treatment exceeds the gain in life expectancy. [J Natl Cancer Inst Monogr 2001;30:109–13]

The Oxford Overview Analysis (1) has clearly demonstrated that adjuvant systemic chemotherapy reduces the risk of mortality for women with both lymph node-positive and lymph node-negative breast cancer and is effective in both premenopausal and postmenopausal women. In light of the proven benefit of chemotherapy, it is reasonable to ask whether there are any groups of breast cancer patients who should not receive this treatment. Potential patient groups who will not achieve a net benefit from chemotherapy include women with an extremely favorable prognosis, where the reduction in mortality from chemotherapy translates to an absolute survival difference of only a few percent; patients in whom the potential risks of chemotherapy outweigh the benefits; and patient subsets for which chemotherapy has not been proven to have a survival benefit. This article will discuss breast cancer patient groups who meet these criteria.

FAVORABLE PROGNOSIS SUBGROUPS

Subsets of lymph node-negative breast cancer patients with a favorable prognosis have usually been defined on the basis of tumor size or histologic subtype. The use of screening mammography has resulted in the increasingly frequent identification of cancers of 1 cm or less in size. Several large datasets (2–6) confirm that tumors of this size have an extremely favorable prognosis. The Breast Cancer Detection Demonstration Project (BCDDP) was one of the earlier studies to report on the favorable outcome of patients with tumors less than 1 cm in size (2). In the BCDDP, the 880 patients with stage I cancer had an 8-year survival rate of 90%, and for those with tumors less than

1 cm in size, the survival rate was 96%. This favorable prognosis was observed for both the interval and screen-detected cancers, where the survival rates at 8 years were 94% and 96%, respectively (2). Eight-year survival rates above 90% were noted across all age groups for tumors of this size, with women aged 40–44 years having a 99% 8-year survival rate compared with 98% for those aged 50–54 years and 94% for those aged 60–64 years.

More recent studies (3–6) have confirmed these favorable survival rates in large, unselected groups of women. The National Cancer Data Base (NCDB) (3), a joint project of the American College of Surgeons Commission on Cancer and the American Cancer Society, has collected data from 1849 hospitals on 240 031 patients who were diagnosed with breast cancer from 1985 to 1991. There were 94 106 patients whose cancer was staged as T1N0, and these women had a 5-year relative survival rate of 95.3%. When this group was broken down further into patients with tumors less than 1 cm and those with tumors between 1 and 2 cm, the relative 5-year survival rates were 98.4% (n = 22 288) and 94.4% (n = 71 818), respectively.

Survival data from two different time periods reported by the Surveillance, Epidemiology, and End Results Program¹ (SEER) are very similar to those of the NCDB. From 1977 through 1982, 57 828 stage I breast cancers were reported (4). The 5-year relative survival rate was 96.3%, ranging from 99.2% for the 269 patients with tumors less than 5 mm in size to 98.3% for those with tumors 0.5–0.9 cm in size and to 85.8% for patients with tumors 1.0–1.9 cm in size. These results are confirmed in the most recent SEER report of patients diagnosed from 1988 through 1994 (5). In this time period, 7842 cancers less than 5 mm in size and 11 543 cancers between 5 and 9 mm in size were reported. Both of these groups had 5-year relative survival rates of 100%. In the National Surgical Adjuvant Breast and Bowel Project (NSABP) protocol B-21 (6), a study of invasive cancers less than or equal to 1 cm in size with negative axillary lymph nodes, patient accrual took place from 1989 to 1998. The 5-year survival rate is 97% for the 1009 patients in the study, regardless of the treatment arm (radiotherapy, radiotherapy plus tamoxifen, or tamoxifen). These studies, using a large convenience sample, population-based data, and patients entered in a breast cancer clinical trial, confirm the favorable prognosis of patients with breast cancers less than 1 cm in size across patient groups.

Long-term follow-up data are available for much smaller numbers of patients with cancers of 1 cm or less in size, since these tumors were infrequently diagnosed clinically. Rosen et al.

Affiliation of authors: Lynn Sage Breast Center and the Department of Surgery, Northwestern University, Chicago, IL.

Correspondence to: Monica Morrow, M.D., Northwestern University, 675 N. St. Clair St., Galter 13-104, Chicago, IL 60611 (e-mail: mmorrow@nmh.org).

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(7) reported a 10- and 20-year disease-free survival (DFS) for 171 patients with cancers of 1 cm or less in size and for 303 patients with cancers between 1.1 and 2.0 cm. In those patients whose tumors were less than or equal to 1 cm in size, the DFS at 10 and 20 years was 91% and 88% compared with 77% and 72%, respectively, for patients whose tumors were 1.1–2.0 cm in size. For women whose tumors were less than or equal to 1 cm in size, the probability of death from breast cancer did not exceed the probability of death from cardiovascular disease, other types of cancer, or other causes.

Single institution studies (8,9) have attempted to identify poor-prognosis subgroups of patients with lymph node-negative cancers on the basis of pathologic features, such as nuclear grade or lymphatic invasion. Leitner et al. (8) reported 218 patients with T1a and T1b disease with lymph node-negative breast cancer, with a median follow-up of 6.9 years. Poor nuclear grade and the presence of lymphatic invasion were found to be significant prognostic factors. Of the 196 patients with complete prognostic information, 20 (10%) had both poor nuclear grade and lymphatic invasion. Relapse-free survival for this group of patients was statistically significantly poorer than for patients with one or neither of these factors. Lee et al. (9) also found histologic grade, lymphatic invasion, hormone receptors, high Ki67, and bcl2 expression to be statistically significant prognostic indicators. However, only seven of the 87 patients in their series developed recurrence, precluding multivariate analysis. In contrast, Rosen et al. (10) were unable to identify a patient subset with a particularly high or low risk of recurrence on the basis of pathologic features. Of the 2288 patients with cancers less than 1 cm in size reported to the NCDB (3), 10312 (46.3%) had information on tumor grade. Five-year survival by grade is shown in Table 1. Even patients with high-grade tumors (3,4) had 5-year survival rates of 96% or greater. The NCDB results are similar to those found by SEER (5) and the Swedish Two-County Mammographic Screening Study (11), where survival rates for patients with tumors less than 1 cm in size approached 100%, regardless of grade.

The datasets cited above (2–7,11) clearly demonstrate a difference in prognosis between stage I cancers of 1 cm or less in size and those between 1 and 2 cm in size. The addition of histologic grade to tumor size for patients with T1c tumors allows the identification of additional patients with an extremely favorable prognosis. A major problem with the use of grade as a prognostic factor is that it is incompletely reported. In the SEER Program, the grade was reported for only 10.4% of the cases in the 1973 report compared with 34.4% of the cases in 1987 and 60.2% of the cases diagnosed from 1988 through 1994 (4,5,12). The SEER data indicate that survival is significantly better for grade 1 cancers, regardless of stage (12), with 5-year survival rates ranging from 93% for grade 1 cancers to

65% for grade 3 cancers. For patients with stage I, grade 1 cancers, 5- and 10-year survival rates were 99% and 95% compared with 94% and 85% for those with stage I, grade 3 cancers. The 10% survival difference at 10 years on the basis of grade is clinically significant and should be used to further stratify a patient's risk of relapse. It is noteworthy that the incidence of grade 1 tumors increases as tumor size decreases. In the SEER dataset, 26% of tumors less than 5 mm in size were grade 1 compared with 12% of those 1.0–1.9 cm in size and only 4% of those between 3.0 and 3.9 cm in size (12). Since screening mammography results in the detection of a greater number of stage I breast cancers, failure to recognize the prognostic importance of grade may result in the overtreatment of increasing numbers of women.

Histologic subtype of breast cancer is another prognostic factor, although it is one that affects a relatively small group of women. The favorable histologic subtypes of breast cancer include tubular carcinoma, mucinous carcinoma, papillary carcinoma, and adenoid cystic carcinoma. Together, these accounted for 4.5% of the 68273 breast cancers reported to SEER in the 1988–1994 interval (5). Rosen et al. (7) observed that the 20-year DFS for patients with breast cancers of these favorable histologic types (plus medullary carcinoma) up to 3 cm in size was 87% compared with 70% for infiltrating ductal and lobular carcinomas (Fig. 1). The favorable prognosis of medullary cancer is not confirmed in all reports, and it will not be considered further (13,14). However, other data support the findings by Rosen et al. (7) for tubular and mucinous cancers (15–21). A literature review of 300 lymph node-negative tubular cancers of all sizes, the majority with long-term follow-up, identified only four relapses (15–20). These studies are summarized in Table 2. The criterion for inclusion in Table 2 was a tumor consisting of 90% tubular elements. In the 1101 tubular cancers reported to SEER (5), the 5-year survival was 100%, regardless of stage.

The SEER data also indicate that patients with mucinous carcinoma have a significantly better prognosis than those with infiltrating ductal carcinoma, independent of stage (21). From 1973 through 1990, 4082 mucinous adenocarcinomas and 139154 infiltrating ductal carcinomas were recorded by SEER (21). Life-table analysis with Cox proportional hazards analyses to adjust for major covariates demonstrated that the relative risk (RR) of death because of breast cancer for women with mucinous carcinoma was 0.38 that of women diagnosed with infiltrating ductal carcinoma (95% confidence interval [CI] = 0.34 to 0.42). Similar to grade 1 lesions, special histologic tumor types are diagnosed more frequently in patients undergoing screening mammography (22,23), again raising the possibility of overtreatment of more women in the future unless the prognostic implications of tumor histology are clearly stated.

SUBGROUPS WITH LACK OF CLEAR EVIDENCE OF BENEFIT VERSUS TOXICITY OF THERAPY

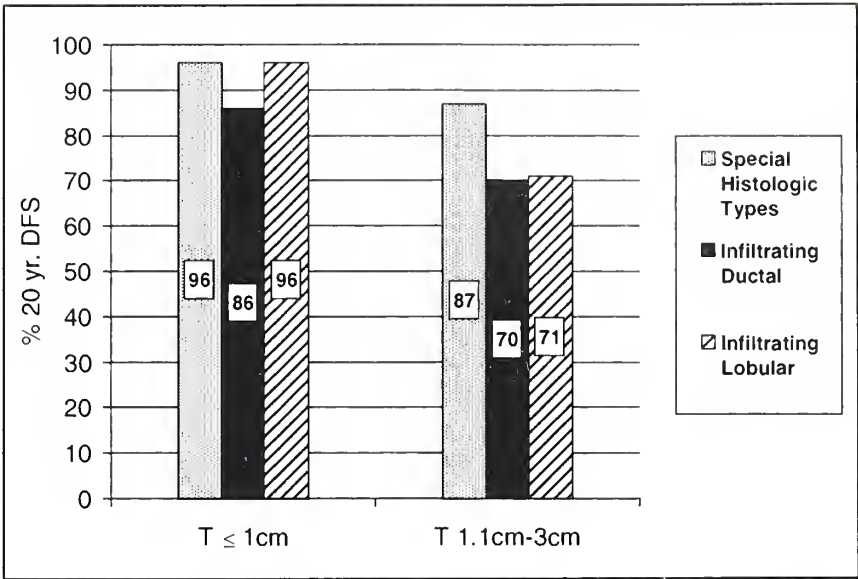
The Oxford Overview Analysis demonstrates a 49% reduction in the annual odds of breast cancer recurrence and a 25% reduction in the odds of death for women with estrogen receptor (ER)-positive, lymph node-negative breast cancer treated with 5 years of tamoxifen (1). Chemotherapy also reduces the risk of relapse and death in all age groups, but the benefit is less for women over age 50 years than for their younger counterparts. The magnitude of the additional survival benefit must be

Table 1. Impact of histologic grade on survival in lymph node-negative breast cancer less than 1 cm in size*

Grade	n	Percentage 5-y survival	SEM
1	2783	98.6	0.8
2	5008	98.2	0.6
3	2338	96.0	0.9
4	183	99.9	2.7

*SEM = standard error of the mean. Data from the National Cancer Data Base (3).

Fig. 1. The effect of tumor histology and size on 20-year disease-free survival (DFS) in patients with lymph node-negative breast cancer. DFS is significantly prolonged in patients with tumors of special histologic types between 1.1 and 3 cm in size compared with those with ductal and lobular carcinoma ($P<.0001$ [data from Rosen et al. (30)]).



weighed against the toxicity of treatment, particularly in the lymph node-negative patient with a relatively favorable prognosis.

The NSABP B-20 trial compared the use of tamoxifen alone with that of tamoxifen plus chemotherapy in women with lymph node-negative, ER-positive breast cancer (24). After 5 years, a 4%–5% improvement in DFS was seen with the addition of chemotherapy, and a subset analysis failed to identify a subset of patients who did not benefit from the addition of chemotherapy. However, only 27% of the study participants were aged 60 years or older, resulting in 201 patients in this age group in the methotrexate, 5-fluorouracil, and tamoxifen (MFT) arm of the study and 207 in the cyclophosphamide plus MFT (CMFT) arm of the study. A comparison of outcome on the basis of tumor size demonstrates that, for patients with tumors with a clinical size of 2 cm or less treated with tamoxifen alone, the event rate per 100 women per year was 2.75, which was reduced to 2.28 and 1.99, respectively, with MFT and CMFT. In comparison, for tumors clinically measured as 2.1 cm or larger in size, the event rates for tamoxifen, MFT, and CMFT were 4.96, 2.83, and 2.69, respectively, per 100 women. An analysis of the risk reduction seen with MFT or CMFT relative to tamoxifen alone on the basis of age is shown in Fig. 2. The magnitude of benefit is clearly greater for women aged 49 years or less. For those 50 years and

older, MFT resulted in only a 10% reduction in the risk of events related to DFS, and a 26% reduction in these events was seen with treatment with CMFT. In both cases, the subset analyses do not demonstrate statistical significance (MFT: RR = 0.90 [95% CI = 0.64 to 1.25]; CMFT: RR = 0.74 [95% CI = 0.52 to 1.05]).

In addition to the relatively small benefits in DFS and overall survival, the addition of chemotherapy to tamoxifen resulted in significant toxicity. Grade 3 or 4 overall toxicity was experienced by 4% of the patients in the tamoxifen group, by 17% in the MFT group, and by 25% in the CMFT group (24). In particular, the addition of chemotherapy to tamoxifen resulted in an increase in the number of thromboembolic events, from 1.8% in the tamoxifen group to 6.5% in the MFT group and to 7.0% in the CMFT group.

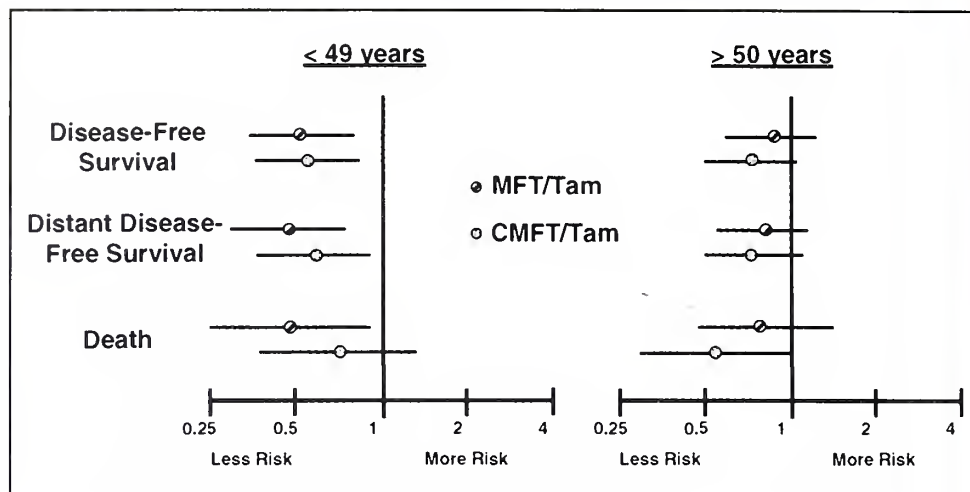
These findings suggest that identification of higher risk subsets of ER-positive, lymph node-negative postmenopausal women would be useful. In an analysis of 4000 breast cancer patients with negative lymph nodes and positive hormone receptors who participated in the NSABP B-14 trial (25), substantial variation in survival in this patient group was identified. Clinical tumor size, age, progesterone receptor status, and ploidy were found to be statistically significant predictors of outcome. Women over the age of 50 years at the time of surgery were found to have an increased risk of death compared with those aged 50 years at the time of surgery, suggesting that this group would experience benefit from the addition of chemotherapy to tamoxifen. However, after censoring other causes of death such as second primary cancers and deaths before recurrence of breast cancer, the rate of treatment failure for women over the age of 50 years was relatively constant (25). This emphasizes the importance of considering an individual woman's competing risks for mortality when considering adding chemotherapy to tamoxifen. Overall, the addition of chemotherapy to tamoxifen is associated with a relatively small survival benefit and results in a moderate increase in toxicity. On the basis of this information, it does not seem reasonable to recommend chemotherapy for all lymph node-negative, ER-positive postmenopausal women. This treatment should be reserved for those at higher risk of breast cancer recurrence, identified by larger tumor size, high S-phase frac-

Table 2. Prognosis in lymph node-negative tubular carcinoma

Author	No. of patients	Size, cm	No. of recurrences
Rose et al. (7)	24	≤2*	0
Cooper et al. (15)	12	1.5†	0
Winchester et al. (16)	40	1.0†	1
Deos et al. (17)	90	0.8†	0
Parl and Richardson (18)	17	1.6‡	1§
Peters et al. (19)	16	1.8‡	0
McDivett et al. (20)	123	0.9‡	2
Total	300		4 (1.3%)

*One T2 case, size not specified.
†Median.
‡Mean.
§Clinically lymph node negative, axilla not dissected.

Fig. 2. Relative risks of disease-free survival, distant disease-free survival, and death in patients treated with methotrexate, 5-fluorouracil, and tamoxifen (MFT) versus tamoxifen alone (Tam) or cyclophosphamide, methotrexate, 5-fluorouracil, and tamoxifen (CMFT) versus Tam in National Surgical Adjuvant Breast and Bowel Project-B20, on the basis of age. Horizontal lines represent 95% confidence intervals. A statistically significant benefit for chemotherapy is only seen in women aged 49 years or younger. [From Fisher et al. (24)].



tion, and negative progesterone receptors, who are at a low risk of death from other causes.

SUBGROUPS WITH LACK OF PROVEN BENEFIT FROM CHEMOTHERAPY

The Oxford Overview Analysis demonstrates no improvement in relapse-free or overall survival in patients aged 70 years and older with ER-negative breast cancers treated with chemotherapy (1). However, only 600 women in this age group were available for analysis. The lack of benefit for chemotherapy has been interpreted by some as a problem of insufficient sample size (26). However, it is equally likely that, if chemotherapy has the same effect in women aged 70 years and older as it does in those aged 50–59 years (11% reduction in the risk of overall mortality), the 2% gain in 10-year survival that would result in lymph node-negative women would be negated by the high rate of death from other causes. This would result in the failure to observe a substantial survival benefit, even in a large group of women.

The 1998 consensus panel at the St. Gallen Breast Cancer Conference recommended that women older than 70 years receive chemotherapy for ER-negative tumors greater than 2 cm in size with negative lymph nodes (27). However, this recommendation is not well supported by the available data. Diab et al. (28) examined the outcome of 4011 breast cancer patients aged 75 years and older who did not receive any adjuvant therapy and were followed a median of 5.3 years. Fifty-three percent of the patients were aged 80 years or older, and 89% had ER-positive tumors. Only 13% of the group had tumors less than 1 cm in size. The 5-year overall survival rate for lymph node-negative patients was 70% compared with 69% for an age- and sex-matched group from the general population. For lymph node-positive patients, the 5-year survival rate was 52% compared with 67% for the general population.

Desch et al. (29) used a Markov model to determine the benefit of adjuvant chemotherapy in hypothetical cohorts of women aged 60–80 years with ER-negative, lymph node-negative breast cancer. The model used a risk of recurrence of 5% per year, or 23% at 5 years, and estimated a 20% reduction in the risk of recurrence with chemotherapy. In a 60-year-old woman, a survival gain of 5.5 months was seen with chemotherapy, falling to 2.8 months after adjustment for quality of life.

For a 75-year-old woman, the survival gain was 2.9 months, or 1.8 months after adjustment for quality of life. The gain in active life expectancy, defined as being fully independent in bathing, dressing, transfer, and eating, for a 75-year-old woman was 0.7 month. Regardless of the calculation used, the gain in life expectancy never exceeded the 6-month duration of chemotherapy. These results are consistent with the findings of Gelber et al. (30), who performed a meta-analysis of quality-adjusted survival on 3920 patients aged 50 years or older with lymph node-positive breast cancer who were treated in randomized trials with chemotherapy plus tamoxifen or with tamoxifen only. Even in this group with a poorer prognosis, a survival gain of only 2 months was observed, which was not statistically significant after adjusting for quality of life. Actual data on the outcome of chemotherapy in elderly women and the impact of comorbidity on outcome are urgently needed to clarify this issue. On the basis of the available data, it is not possible to conclude that all ER-negative, lymph node-negative breast cancer patients over age 70 years will benefit from treatment. Therapy should be reserved for the woman with large T2 tumors in good overall health, where breast cancer is clearly the major risk factor for mortality.

CONCLUSIONS

At present, chemotherapy is administered routinely to women with lymph node-negative breast cancers greater than 1 cm in size. Use of the 1-cm cutoff appears to be appropriate, since a group of women with tumors less than 1 cm with a poor prognosis has not been identified in large datasets. Increased recognition of the extremely favorable outcome and the corresponding lack of a major benefit from chemotherapy are needed for women with lymph node-negative breast cancers 1–2 cm in size that are grade 1 and for those with tubular and mucinous cancers up to 3 cm in size. With the increased use of screening mammography, these cancers will make up a greater percentage of the breast cancer burden, increasing the need for consideration of their prognostic implications.

In women with ER-positive, lymph node-negative breast cancer, the benefit of adding chemotherapy to endocrine therapy is small and is often outweighed by added toxicity. Improved definition of prognostic subsets and stratification of outcomes on the basis of comorbidity are needed to better define therapeutic

recommendations in this diverse subset of women. In women aged 70 years and older with lymph node-negative breast cancer, death from causes other than breast cancer is a major issue. A survival advantage for chemotherapy has not been clearly demonstrated and, if present, is likely to be quite small. Treatment in this group should be reserved for those women at high risk of relapse from breast cancer in whom major comorbidities are absent. Philosophically, this is a substantially different recommendation than advocating therapy in older women unless major comorbidity is present. Ultimately, the decision to have chemotherapy is made by an individual patient after a discussion of the risks and benefits of treatment in her individual case. For some patients, an improvement in survival of 1%–2% is meaningful, and they will opt for chemotherapy, even if they fall into the favorable prognostic groups discussed above. However, the available data do not justify mandating chemotherapy for all patients in the categories discussed in this article.

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NOTES

¹*Editor's note:* SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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Overview of the Six Available Randomized Trials of High-Dose Chemotherapy With Blood or Marrow Transplant in Breast Cancer

Karen H. Antman

In animal models of breast cancer and other malignancies, dose of chemotherapy correlates with curative therapy, while cumulative dose is associated with survival (1). Thus, using high doses when cure is the objective, but using smaller doses over a prolonged period when palliation and survival are the goal may be appropriate strategies.

Of the 11 randomized trials of high-dose therapy in breast cancer reported to date (Table 1), five included women with metastatic cancer. Of the six remaining adjuvant studies, one, the South African study, has been discredited (2). Two of the remaining studies randomly assigned fewer than 100 patients. Thus, neither one could exclude a survival difference of 30% or less. The Scandinavian study does not compare high-dose therapy with conventional-dose therapy. The two remaining moderately large trials include the Dutch and American intergroup studies with 885 and 785 patients, respectively.

Mortality was consistently low, in the 0%–2.5% range, for the high-dose regimens except for the carmustine (BCNU)-containing Cancer and Leukemia Group B (CALGB)/Intergroup study, which had a 7.4% toxic mortality rate. Mortality for the more conventional dose arms was in the range of 0%–1%.

The Dutch trial is the largest of all published studies (885 randomly assigned patients) and, therefore, has the greatest statistical power to detect modest differences (3). It compares four courses of FEC (i.e., a combination of 5-fluorouracil, epirubicin, and cyclophosphamide) with either an additional cycle of FEC or with CTCb (i.e., a combination of cyclophosphamide, thiotepa, and carboplatin) with stem cell support followed by surgery, radiation therapy, and tamoxifen for 2 years. In the study as a whole, the mortality was one of 443 patients on standard-dose FEC and four of 442 on high-dose CTCb. At a median of 3 years follow-up, a trend ($P = .057$) has emerged in disease-free survival (DFS) favoring high-dose therapy. In a planned analysis of the first 284 patients, at a median follow-up of 6 years, disease-free survival and overall survival were significantly better for the high-dose therapy.

The CALGB/Intergroup study compares high with intermediate-dose cyclophosphamide, BCNU, and cisplatin (CBP) after four cycles of a CAF (i.e., a combination of cyclophosphamide, doxorubicin, and 5 fluorouracil) induction (4). Although a critique of the study is that intermediate-dose CBP is not a standard regimen, scientifically, this design is a pure comparison between high- and intermediate-dose CBP.

This first-generation BCNU-containing regimen had a 7.4% mortality, which varied with the experience of the transplant center and increased with patient age. Pulmonary and hepatic toxicity was also substantial. With a median of 5.1 years of follow-up at the time of presentation at the consensus meeting, differences are not statistically significant in either progression-free survival or overall survival between the two groups. Fewer events have occurred than would have been predicted from his-

torical series, suggesting either patient selection or an effect of the "intermediate"-dose CBP. Importantly, fewer relapses have occurred in the high-dose arm, but survival in the two arms is similar, presumably because of the early toxic mortality of 7.4%.

The Scandinavian trial compared three cycles of induction FEC followed by one high-dose cycle of CTCb with nine cycles of moderately high dose "tailored" FEC. (That is, the doses in mg/m^2 of FEC were tailored to individual tolerance up to 600 of 5-fluorouracil, 120 of epirubicin, and 1800 of cyclophosphamide per cycle.) The cumulative doses for tailored therapy that were actually delivered substantially exceeded those for the CTCb arm. Therefore, this study assesses the role of one high-dose cycle compared with nine cycles of chemotherapy intensified to individual tolerance with a higher cumulative chemotherapy dose. The study probably includes some patients with metastatic disease, since patients with involved marrow and abnormal bone scans were included (5).

At a median follow-up of just 3 years, disease-free survival is significantly improved for the six cycles of tailored-dose therapy compared with one high-dose cycle. Of the 251 patients on the tailored-dose arm, six developed leukemia and three developed myelodysplasia, compared with none on the marrow transplant arm. Because the median follow-up is only 3 years, additional cases are possible or even likely. Topoisomerase-associated leukemias tend to occur early, but alkylating agent-associated leukemias would emerge later than the current median follow-up. Stem cells collected after three cycles of chemotherapy for use in stem cell support may be less damaged than those exposed *in situ* to nine chemotherapy cycles escalated to patient tolerance.

The South African study was reported to be a direct comparison of conventional CAF versus two cycles of high-dose chemotherapy (without a preceding induction phase) (2). An independent audit team documented many inconsistencies in eligibility criteria, as well as in reported data. Documentation of treatment and outcome for the control group was totally unavailable. The title of the protocol provided to the audit team, however, suggests that the control group was treated with cyclophosphamide, mitoxantrone, and vincristine and not CAF. On the basis of these findings, the abstract has been withdrawn and the data are best considered unreliable (6).

The Netherlands Cancer Institute randomly assigned 81 women with an involved apical axillary lymph node after four initial courses of FEC either to an additional cycle of FEC or to CTCb with stem cell support followed by surgery, radiation therapy, and tamoxifen for 2 years. At a median follow-up of

Affiliation of author: Department of Medicine and Pharmacology, Columbia University, and Herbert Irving Comprehensive Cancer Center, New York, NY.

Correspondence to: Karen H. Antman, M.D., Columbia University, MHB 6N-435, 177 Fort Washington Ave., New York, NY 10032 (e-mail: kha4@columbia.edu).

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Table 1. Randomized adjuvant high-dose breast cancer studies*

	No. randomized	% toxic deaths		Med y FU	% 3-y EFS		P	% 3-y survival		P	Reference
		HDC	Control		HDC	Control		HDC	Control		
Dutch phase 3	885	0.9	0.2	3.5	72	65	.057	84	80	.31	(4)
First 284-patient subset	284	NA	NA	7.0	77	62	.009	89	79	.039	(4)
CALGB Intergroup	783	7.4	0	3.6	71	64	NS	79	79	.29	(5)
Dutch phase 3 pilot	81	0	0	4.1	70	65	.97	82	75	.84	(6)
The University of Texas M. D. Anderson Cancer Center	78	2.5	0	6.5	48	62	NS	58	77	NS	(7)
Study of one versus six high-dose cycles Scandinavian	525	0	0.7	3.0	72	63	0.012	83	77	.12	(8)

*Statistically significant differences are shown in bold. The high-dose arm of the Scandinavian study is the "tailored" arm and is compared with one cycle of high-dose chemotherapy with BMT as the control. The South African adjuvant study has been discredited and is not shown (3). BMT = bone and marrow transplant; EFS = event-free survival; HDC = high-dose chemotherapy; NA = not available; NS = not significant; Med y FU = median years follow-up; CALGB = Cancer and Leukemia Group B.

Table 2. Ongoing or unpublished randomized adjuvant high-dose breast cancer trials*

Eligible No. of LN+	Chair	Group	Accrual target (No. of patients)	Accrual (No. of patients)
>3	Leanard	UK, Anglo-Celtic	604	Closed
>3	Gianni	Milan Cancer Institute	350	Closed
>4	Russel/Nabholtz	International BCIRG	460	Still accruing; at approximately 290
>4	Bearman	Intergroup	1000	Closed at approximately 500
>7	Roche	Pegase 01	314	Closed
>8	Zander	German study	307	Closed
>9	Nitz	German group	400	Accruing
>9	Basser	Australia, IBCSG	340	Closed
>9	Tallman	ECOG	550	Closed
Stage 3	Seeber	German group		Closed

*Adjuvant trials by number of involved axillary lymph nodes (LN+). BCIRG = Breast Cancer International Research Group; IBCSG = International Breast Cancer Study Group; ECOG = Eastern Cooperative Oncology Group.

49 months, DSF and overall survival were similar. Although this randomized phase II study is mature, in that most of the expected events have already occurred, this small study cannot exclude differences of less than 30% in survival (7). Thus, the Dutch undertook their larger study, described above, which currently shows about a 15% advantage in DFS for high-dose therapy in the group with the longest follow-up.

A second small study at The University of Texas M. D. Anderson Cancer Center, Houston, randomly assigned 78 patients to eight cycles of FAC with or without two cycles of high-dose chemotherapy with cyclophosphamide, etoposide, and cisplatin. Three patients randomly assigned to conventional-dose therapy received transplants elsewhere; six patients randomly assigned to receive a transplant did not receive it. With a median follow-up exceeding 78 months, no advantage for high-dose chemotherapy has emerged. This study was closed because of slow accrual, but it has the statistical power to rule out differences in outcome of more than 30% (8).

Ongoing or unpublished randomized high-dose therapy studies in breast cancer are shown in Table 2.

SUMMARY OF RANDOMIZED ADJUVANT TRIALS

On the basis of the data so far, mortality is 0%–2% in all studies but one—about the same as for conventional dose therapy. The South African adjuvant study has been discredited. The Scandinavian study, which compares one high-dose cycle with six cycles of escalated dose tailored to individual tolerance,

does not compare conventional- with high-dose chemotherapy. Two small studies, comparing standard FAC or FEC with or without high-dose chemotherapy, report no differences but can not exclude a 30% difference. In fact, one was the pilot study for the larger Netherlands trial, which currently has a trend in favor of high-dose therapy, with statistically significant differences in DFS and overall survival in the first 285 patients accrued with 6 years of follow-up. These two Dutch studies provide an object lesson in biostatistics: specifically, the issue of drawing conclusions from underpowered studies. The U.S. study, comparing high-dose therapy with an intermediate-dose therapy, has a statistically significant decreased relapse rate for the high-dose arm—a biological effect similar to that in the Dutch study. However, the higher mortality obviates any advantage of the high-dose therapy.

Survival curves for conventional adjuvant therapy of breast cancer show no plateau indicating cure for 15–20 years after diagnosis (9). Additional follow-up of the two larger randomized trials that compare high-dose with relatively conventional-dose chemotherapy and the completion of other ongoing randomized trials will provide more reliable information to determine what role high-dose chemotherapy regimens should have, if any, in the management of high-risk primary breast cancer.

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Treatment Guidelines and Techniques in Delivery of Postmastectomy Radiotherapy in Management of Operable Breast Cancer

Lori J. Pierce

Radiation therapy has been shown to statistically significantly reduce the risk of locoregional recurrence in high-risk patients with operable breast cancer following mastectomy and systemic therapy. Recent trials have also demonstrated a significant survival benefit following radiotherapy in high-risk patients. Therefore, it is important to identify the patients who could potentially derive that survival benefit and to not offer treatment to those patients who are not at increased risk for failure. Established risk factors that predict for increased rates of locoregional recurrence include axillary lymph node involvement and T3 (or T4) disease. While treatment-related factors, such as the extent of the axillary dissection and extent of lymph nodal positivity, also undoubtedly affect locoregional recurrence, additional studies are needed to define the magnitude of their risk. Locoregional patterns of failure have identified the chest wall and supraclavicular/infraclavicular regions to be the most common sites of locoregional failure following mastectomy, which justifies treatment to these regions. While long-term complications are uncommon following locoregional radiotherapy, careful treatment planning is critical to minimize cardiac (and pulmonary) toxicity. [J Natl Cancer Inst Monogr 2001;30:117-24]

INTRODUCTION

Virtually every retrospective and prospective study examining the effect of radiotherapy (RT) following mastectomy has demonstrated a statistically significant reduction in the risk of locoregional recurrence by the addition of postmastectomy RT (PMRT) (1-15). In the recent update by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) of the overview of 40 randomized trials, a highly statistically significant two-thirds reduction in locoregional recurrence was observed following RT (two-sided $P < .0001$) (16).

With the significant benefit of RT on locoregional control (LRC), attention is now focused on the effect optimal LRC has on survival. The EBCTCG overview demonstrated a statistically significant reduction in breast cancer deaths for patients randomly assigned to receive RT, with an absolute difference of 4.8% (two-sided $P = .0001$); overall survival was of borderline statistical significance in favor of RT, with 20-year survival rates of 37.1% with RT versus 35.9% among control subjects (two-sided $P = .06$) (16). A statistically significant survival advantage was seen with the use of PMRT following chemotherapy and mastectomy in two randomized trials from Denmark and British Columbia (12,14), and a second Danish trial (15) found a statistically significant survival benefit with RT and adjuvant tamoxifen. Specifically, a 9% absolute survival benefit at 10 years was demonstrated in the Danish premenopausal trial (82b) for patients randomly assigned to receive RT following mastec-

tomy and cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), compared with CMF alone ($P < .001$) (14). An identical survival benefit was observed following RT and CMF after 15 years in the smaller British Columbia trial ($P = .02$) (12). In the Danish Breast Cancer Cooperative Group (DBCCG) postmenopausal trial of mastectomy and tamoxifen (82c) (15), a 9% absolute survival benefit was seen at 10 years for women randomly assigned to receive RT ($P = .03$). In a meta-analysis by Whelan et al. (17) of RT trials following mastectomy and systemic therapy, a statistically significant reduction in mortality was observed with RT, with an odds ratio of 0.83 ($P = .004$). Although this analysis was strongly influenced by the positive results of the large premenopausal and postmenopausal Danish trials, it also included the results of 16 other trials published between 1967 and 1999. Thus, these data demonstrate a systemic benefit of postmastectomy RT in high-risk breast cancer patients and emphasize the importance of defining the patients who could potentially derive that survival benefit. This article will review existing selection criteria for postmastectomy RT and will discuss technical aspects and treatment guidelines.

RISK FACTORS FOR LOCOREGIONAL RECURRENCE

Involvement of the axillary lymph nodes is a powerful predictor for locoregional recurrence following mastectomy and chemotherapy in both retrospective and prospective series, with increasing rates of locoregional failure (LRF) associated with increasing lymph node involvement (18-21). In an analysis of four randomized trials conducted by the Eastern Cooperative Oncology Group (ECOG) using adjuvant methotrexate-based regimens, the risk of LRF with or without distant failure was 12.9% in patients with one to three positive lymph nodes and 28.7% for patients with four or more lymph nodes positive at 10 years (19). A series by Stefanik et al. (20), which also studied patients treated with methotrexate-based therapy, demonstrated 5-year rates of actuarial LRF of 9% with one to three positive lymph nodes, compared with 36% with four or more positive lymph nodes. Similar results were recently reported from The University of Texas M. D. Anderson Cancer Center, Houston, with doxorubicin-based therapy, where 10-year actuarial rates of isolated LRF and total LRF were 10% and 14%, respectively, for one to three positive lymph nodes; 21% and 25%, respectively, for four to nine positive lymph nodes; and 22% and 34%, respectively, for 10 or more positive lymph nodes (21).

Although many trials have randomly assigned women to RT following chemotherapy and mastectomy, only a few have reported LRF results by the number of positive axillary lymph

Correspondence to: Lori J. Pierce, M.D., Department of Radiation Oncology, University of Michigan School of Medicine UHB2C490, Box 0010, 1500 E. Medical Center Dr., Ann Arbor, MI 48109 (e-mail: ljperce@umich.edu).

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nodes (Table 1). In general, the incidence of LRF in the absence of RT in most U.S. series has been 5%–15% in patients with one to three positive lymph nodes and 20%–50% in patients with four or more positive lymph nodes at 10 years. As shown in Table 1, the rates of LRF in the British Columbia and Danish trials (12,14,15) exceed these estimates and have caused some to question the extent of the surgical resection in these trials. The median number of axillary lymph nodes dissected was seven in the Danish trials, resulting in increased rates of axillary failure without RT (i.e., 13% axillary failure as first failure in surgical controls versus 2% following RT) (22). The limited axillary surgery may not have adequately identified those patients with one to three positive lymph nodes from those with four or more positive lymph nodes, thus reporting rates of failure usually seen with four or more positive lymph nodes in patients with one to three involved lymph nodes following inadequate dissection (23). These limitations in the data should be considered when incorporating the information learned in the Danish trials into practice guidelines for patients with one to three involved axillary lymph nodes.

Tumor size also appears to be an independent risk factor for LRF following mastectomy, although data are limited in the lymph node-negative population. In the randomized trial by Klefstrom et al. (24) in stage III disease, patients with T3N0 breast cancer treated with RT with or without vincristine, doxorubicin, cyclophosphamide, and levamisole had a 7% risk of LRF as a component of first failure compared with a 38% risk without RT. In the series by Katz et al. (21), the 10-year actuarial isolated rate of LRF with T3 disease and negative lymph nodes was 29%, compared with 6%–11% with smaller lymph node-negative lesions; only seven patients, however, had T3N0 disease in this series. In a larger retrospective series of 101 patients with T3N0 disease (median tumor size of 6 cm), 15% of the patients developed LRF as a component of first failure without RT with a 7.8-year follow-up (25). Many series (14,15,19,21,26) have reported the risk of LRF using combinations of tumor size and lymph node involvement. In the ECOG analysis by Recht et al. (19), the risk of LRF in the group with one to three positive lymph nodes increased from 12% to 31% for patients with T1/2

versus T3 lesions and from 20% to 45% in the group with four to seven positive lymph nodes at 10 years. Thus, data from both lymph node-negative and lymph node-positive disease demonstrate increased risk of LRF with T3 disease.

Rates of LRF for other tumor- and treatment-related factors, including estrogen receptor status, tumor grade, S-phase, lymphatic invasion, p53 accumulation, multicentricity, and margin status, have been reported (13,26–32). Conflicting results and limited data have prevented clear assessment of risk, and more studies are needed to establish a consensus. Two additional factors, the presence of extracapsular extension (ECE) and the number of lymph nodes examined, have been more extensively studied and warrant further comment. Reports (33–35) have demonstrated increased rates of LRF and distant failure in patients with microscopic ECE. However, ECE is correlated with increased risk of axillary lymph node involvement (35–37). Donegan et al. (35) reported the presence of ECE in 39% of cases with one to three positive lymph nodes, 78% with four to seven positive lymph nodes, and 92% with eight or more positive lymph nodes. A patterns-of-failure study by Mignano et al. (34) found the chest wall to be the most common site of LRF in patients with ECE, with 16% of patients having a chest wall recurrence and no failures at the axilla or other regional sites. Thus, ECE does not appear to predict for a statistically significantly increased risk of axillary recurrence, and correlation with increasing lymph node involvement may account for chest wall risk.

The number of positive axillary lymph nodes and the number of lymph nodes examined appear to affect the cumulative incidence of the sites of LRF (19). Specifically, in patients with one to three positive lymph nodes, a statistically significantly greater number of axillary failures was observed in the ECOG data when only two to five lymph nodes were examined, compared with when six to 10 and 11 or more lymph nodes were dissected ($P = .0009$). This compared with a statistically significantly greater risk of supraclavicular failure, and not axillary failure, in patients with four or more positive lymph nodes and four to five lymph nodes examined versus six to 10 and 11 or more lymph nodes removed ($P = .02$). Therefore, the extent of axillary resection in conjunction with the number of positive axillary nodes appears to affect the patterns of LRF and should be considered when reporting LRF results.

In summary, accepted risk factors for locoregional recurrence following mastectomy for operable breast cancer include four or more positive axillary lymph nodes and T3 tumor size. ECE does not appear to be an independent predictor of LRF when extent of lymph node positivity is considered. There is no consensus in patterns of recurrence for other tumor-related factors. Further studies are needed. The extent of dissection and the extent of lymph nodal positivity appear to affect patterns of regional failure and should be considered in future studies of locoregional recurrence.

IMPACT OF LOCAL CONTROL ON SURVIVAL

As discussed previously, optimal locoregional control with RT has resulted in statistically significant gains in survival. While it is reasonable to assume that patients at high risk for LRF would be the patients most likely to derive a survival benefit, some trials suggest that it is patients at lower risk for LRF who derive the greatest survival benefit. It has been postulated that it is patients with the least systemic burden who can be

Table 1. Risk of locoregional recurrence with and without radiotherapy (RT) by number of positive axillary lymph nodes*

Trial, No. of positive lymph nodes	No. of patients	Systemic therapy	Locoregional failure		Follow-up, y
			No RT	RT	
Dana-Farber Cancer Institute (9)					5
1–3	83	CMF	5	2	
≥4	118	CA	20	6	
DBCG 82b (14)					9.5
1–3	1061	CMF	30	7	
≥4	510	CMF	42	14	
SECSG (30)					10
≥4	270	CMF	23	13	
DBCG 82c (15)					10.3
1–3	794	Tamoxifen	31	6	
≥4	448	Tamoxifen	46	11	
British Columbia (12)					15+
1–3	183	CMF	20	8	
≥4	112	CMF	51	17	

*DBCG = Danish Breast Cancer Group; SECSG = Southeast Cooperative Study Group; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; CA = cyclophosphamide, doxorubicin.

successfully managed with systemic therapy in whom sterilization of residual locoregional disease (foci for potential dissemination) could have the greatest impact. The 1997 results of the British Columbia trial demonstrated both statistically significant benefits in survival free of systemic disease and breast cancer-specific survival and borderline statistically significant benefit in overall survival following RT (12). With 2 years of additional follow-up, overall survival was statistically significantly improved following RT, with a greater relative reduction in mortality in the group with one to three positive lymph nodes versus the patients with four or more positive lymph nodes (38). In a combined analysis of DBCCG 82b and 82c in patients with at least eight lymph nodes examined, results also indicated that the greatest survival benefit occurred in patients with the least tumor burden. Survival was greatest following RT in women with smaller tumors and fewer positive lymph nodes compared with women with larger tumors and many involved lymph nodes (Overgaard M: personal communication).

The results of the Danish and Canadian trials would suggest that all lymph node-positive patients with operable breast cancer should receive RT to improve breast cancer-specific and overall survival. However, given the surgical limitations of these studies, particularly the Danish trials, caution should be used with generalizing these recommendations to U.S. practice. This is evident particularly in the group with one to three positive lymph nodes, in whom the risk of LRF was 30% in DBCCG 82b (14) compared with a 13% risk recently reported from the U.S. ECOG trials (19). Because of these concerns, a new intergroup trial, sponsored by the Southwest Oncology Group, has recently opened in this country, randomly assigning women with one to three positive axillary lymph nodes to RT or observation following mastectomy and adjuvant chemotherapy (Fig. 1). Patients must have a minimum of 10 lymph nodes dissected. The target accrual is 2500 women over a 5-year period, which is powered to detect a hazard ratio of 1.33, which corresponds to a 5.5% survival difference at 10 years. Primary endpoints will be both overall survival and disease-free survival, with a secondary endpoint of locoregional control.

TREATMENT GUIDELINES FOR OPTIMAL LOCOREGIONAL CONTROL

Results from adjuvant therapy trials that have studied the effect of systemic therapy on locoregional control have been contradictory. While some (39–41) have shown a reduction in the risk of LRF with chemotherapy and tamoxifen, others (42,43) have shown essentially no effect on local control. Perhaps the most comprehensive data can be obtained indirectly

from the overview (16). In the published analysis of the proportional effect of RT on isolated local recurrence (16), RT produced essentially identical reductions in LRF in patients treated with and without chemotherapy and/or tamoxifen, suggesting that systemic therapies had very little effect on the proportional reduction of locoregional recurrence.

Locoregional patterns of failure without RT identify the chest wall as the most common site of failure. As shown in Table 2, over half of all LRF occur at the chest wall, with the mastectomy scar and the surrounding skin at greatest risk for recurrence (44). The second most common locoregional site following level I–II axillary dissection in most series is the supraclavicular and infraclavicular region, with as many as 41% of all LRF occurring in this region (19,21,45,46).

U.S. surgical series of lymph node-positive patients (19,21) have shown the absolute risk of axillary failure to be 2%–4% at 10 years following level I–II axillary dissection. Therefore, full axillary RT is not generally recommended following an adequate dissection. The lower axillary bed, in the region of the tail of the breast, is included in the chest wall fields, and the supraclavicular field includes the apex of the axilla (the infraclavicular region). The remaining portion of the previously dissected axilla would also be irradiated in a full axillary field, which could increase the risk of arm edema, as discussed below. For these reasons, routine full axillary irradiation is generally discouraged following adequate axillary surgery.

The question of whether to incorporate the internal mammary lymph nodes (IMNs) in the RT port has been, and continues to be, vigorously debated. Although previous surgical studies that included internal mammary lymph node dissection have demonstrated pathologic involvement in up to 37% of lymph node-positive breast cancers with inner or central primary tumors (47), clinical evidence of recurrence, as shown in Table 2, is low. Thus, treatment to the IMNs is difficult to justify on the basis of regional patterns of failure. It should be noted, however, that follow-up imaging studies of the IMNs to detect IMN recurrence are not done routinely; therefore, IMN failures may be substantially underreported.

Multiple randomized trials have been performed comparing prophylactic IMN irradiation or resection with observation; the largest of these trials are shown in Table 3. Although some trials have shown trends in favor of patients treated to the IMNs (2,4,48,49), no statistically significant benefit in disease-free or overall survival has been demonstrated to date, but follow-up has been limited in recent trials. Previous studies (18) have shown that 50% of parasternal recurrences occur 10 years or more following surgery, so long-term follow-up is needed to determine rates of IMN failure. Subset analyses (48,50) have suggested statistically significant survival benefits in lymph node-positive patients with medial/central primary tumors. These analyses, however, are subject to criticism of subgroup selection. Therefore, to study the value of internal mammary and medial supraclavicular (SCV) RT with contemporary RT techniques and systemic therapies, the European Organization for Research and Treatment of Cancer (EORTC) has sponsored a trial, randomly assigning women with positive axillary lymph nodes or negative lymph nodes with medial/central lesions to IMN and medial SCV RT or no RT to the IMN and SCV regions. The endpoints of the study are time to locoregional recurrence, distant metastases, and death. To detect a 5% difference in survival, a total of 3780 patients will be randomly

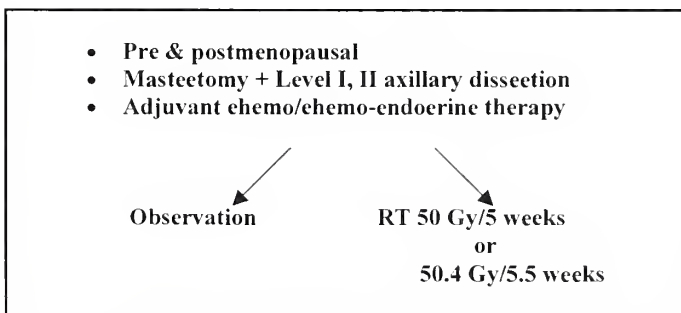


Fig. 1. Schema of the Southwest Oncology Group (SWOG) 9927 postmastectomy radiotherapy trial.

Table 2. Locoregional failure patterns of breast cancer recurrence after mastectomy*

Study	No. of patients	Chest wall (%)	Supra/intra clavicular (%)	Axilla (%)	Internal mammary (%)
ECOG (19)	420	244 (58)	158 (38)	82 (20)	4 (1)
Mallinckrodt Institute of Radiology (45)	224	156 (70)	54 (24)	28 (12)	21 (10)
University of Pennsylvania (46)	128	106 (83)	24 (19)	14 (11)	4 (3)
The University of Texas M. D. Anderson Cancer Center (21)	124	122 (98)	51 (41)	21 (17)	—
Mt. Sinai Medical Center (84)	124	95 (77)	14 (11)	26 (21)	13 (10)

*ECOG = Eastern Cooperative Oncology Group.

Table 3. Results of randomized trials comparing internal mammary lymph node prophylaxis to observation*

Authors	No. of patients	% disease-free survival		% overall survival		F/U, y
		Rx	Obs	Rx	Obs	
Romestaing et al. (85)	1281	83	80	81	81	5.4
Morimoto et al. (86)	192	83	87	92	93	5
Meier et al. (48)	112	—	—	74	60	10
		Central/medial tumors		86	60 ($P = .03$)	—
Fisher et al. (B04) (2)	717	48	42	59	54	10
Host et al. (4)†						
Oslo II, stage I	356	71	78	77	81	10
Oslo II, stage II	186	57	43 ($P = .04$)	58	53 ($P = .15$)	10
Lacour et al. (49)	1453	56	51	56	53	10
Institut Gustav-Roussy (55)		N+ central/medial tumors		53	28 ($P = .05$)	15
Palmer and Ribeiro (87)						
Lymph node negative	281	—	—	16	26	30
Lymph node positive	460	—	—	8	8	30
Veronesi et al. (88)	737	—	—	21	20	30

*Rx = radiotherapy or surgical prophylaxis; Obs = observation; F/U = follow-up.

†Values extrapolated from actuarial curves.

assigned to receive treatment. A second open trial sponsored by the National Cancer Institute of Canada is also studying the value of regional irradiation in patients treated with breast conservation. Patients must have either tumors with positive lymph nodes or high-risk lymph node-negative disease designated as T3 disease or T2 primary tumors with fewer than 10 lymph nodes removed and a primary lesion that is either ER negative or high grade or has evidence of lymphovascular invasion. The random assignment is RT to the breast only or to the breast and regional lymph nodes, i.e., internal mammary, supraclavicular, ± axillary regions. Endpoints will be overall survival, disease-free survival, isolated locoregional disease-free survival, and distant disease-free survival. The desired accrual is approximately 1800 patients over 4 years.

In summary, on the basis of current evidence, locoregional radiation following mastectomy is recommended for patients at high risk of recurrence. Radiation should be delivered to the areas most at risk, including the chest wall, supraclavicular lymph nodes, and axillary apex when more than four axillary lymph nodes are involved. Full axillary RT is not routinely recommended to an adequately dissected axilla. Consensus has not been reached regarding treatment to the internal mammary region. This decision is left to the discretion of the treating radiation oncologist while trials designed to determine the value of treatment are in progress.

POTENTIAL COMPLICATIONS OF TREATMENT

Potential long-term complications following RT and chemotherapy after mastectomy include rib fracture, brachial plexopa-

thy, arm edema, pneumonitis, cardiac effects, and second malignancies. Each will be discussed briefly.

The risk both of rib fracture and of brachial plexopathy is extremely low. Rib fractures occur in less than 3% of patients treated to the breast and chest wall, with a median time to fracture of approximately 12 months (51). In a series from the Joint Center for Radiation Therapy (JCRT), the incidence of rib fracture correlated with machine energy and breast dose, with statistically significantly more fractures occurring with 4-MV beams compared with 6- or 8-MV beams and with doses greater than 50 Gy (51). Chemotherapy increased the rate of rib fracture when the breast dose was less than 50 Gy. In all cases, fractures healed without intervention. Permanent brachial plexopathy from radiation to a supraclavicular and axillary apex field occurs in less than 1% of patients with doses less than or equal to 50 Gy in 2-Gy fractions. Factors shown to increase the incidence of plexopathy are the use of a supraclavicular field, an axillary dose greater than 50 Gy, the use of chemotherapy (51), and daily fractions in excess of 2 Gy (52).

Multiple series have shown that the extent of axillary surgery and regional irradiation after axillary dissection are treatment-related risk factors that affect the risk of arm edema (53–57). Researchers from the Royal Marsden Hospital, Sutton Surrey, UK, found that the risk of subjective lymphedema was 8% after axillary RT only, 9% after axillary sampling and axillary RT, 7% after axillary clearance only, and 38% after axillary clearance and RT ($P < .001$) (53). Larson et al. (56) also reported a 37% risk of arm edema following full dissection and axillary RT. In a complication analysis of the Danish postmastectomy trials, Højris et al. (55) reported a statistically significant in-

crease in the subjective assessment of lymphedema following full axillary RT and partial axillary dissection compared with surgical controls. In radiation series where regional treatment has been restricted to a supraclavicular and axillary apex field following a level I–II axillary dissection, rates of edema have been low (58). Pierce et al. (58) from the University of Michigan, Ann Arbor, reported a 3% rate of arm edema after limited supraclavicular and infraclavicular RT. Therefore, full axillary RT should be discouraged following complete level I–III axillary dissection. Limitation of axillary RT to the apex of the axilla following level I–II dissection appears to minimally increase the risk of edema beyond that observed following surgery only.

The overall risk of pneumonitis is approximately 5% and is generally transient (59). The risk increases, however, with increasing lung volume in the tangent fields and with treatment to the supraclavicular, axillary apex, and internal mammary regions (59,60). The risk also appears to increase with the use of concurrent chemotherapy. Lingos et al. (59) found the risk of pneumonitis to be 1.3% for patients who were treated to an SCV field and who received sequential chemotherapy versus 9% for those receiving concurrent chemotherapy ($P = .002$). Therefore, although the overall risk of pneumonitis is low, the increase observed with concurrent chemotherapy and RT should be considered when contemplating sequencing strategies of locoregional and systemic therapies.

Potential radiation-induced second malignancies after locoregional RT include the development of in-field sarcomas, lung cancer, leukemia, and contralateral breast cancers, all of which are rare. The risk of developing a sarcoma in the RT field has been estimated by Kurtz et al. (61) from the Marseilles Cancer Institute, France, to be nine cases per 100 000 patient-years. Ten-, 20-, and 30-year actuarial estimates reported by Taghian et al. (62) from the Institut Gustav-Roussy, Villejuif, France, were 0.2%, 0.43%, and 0.78%, respectively.

Modest increases in the absolute rate of lung cancers have been observed following RT (63). Neugut et al. (64) found a relative risk (RR) of 2.0 among patients treated with RT who survived 10 or more years from diagnosis. A case-control study using Connecticut Tumor Registry data demonstrated a statistically nonsignificant increase in lung cancer cases in nonsmokers who received RT and a statistically significant increase among treated smokers (63,64). A study by Inskip et al. (65) found an RR of 2.8 of lung cancer among patients who received RT for breast cancer at least 10 years earlier. In absolute terms, nine cases of radiation-induced lung cancer would be expected in 10 000 women surviving at least 10 years. Therefore, the absolute number of women potentially affected with radiation-induced lung cancer is very low. However, smoking cessation interventions, which could further reduce this incidence, should be encouraged strongly.

The association of RT with the risk of acute nonlymphocytic leukemias (ANLL) appears to be related to both volume of bone marrow in the irradiated field and dose delivered (66). In a review by Shapiro and Recht (66), the RR of ANLL appeared to be 0.86–3.7 after RT, compared with an RR of 1.3–11.5 after chemotherapy. A higher RR was demonstrated in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-04 and B-06 trials, with a 10-fold RR following RT (and a 24-fold RR following chemotherapy) (67). This represented, however, only four cases, compared with the expected incidence of 0.39. Therefore, collectively, the absolute risk of ANLL associated

with RT is very low, especially with limited bone marrow in the radiation port.

Limited data suggest a slight excess of contralateral breast cancers following RT above the expected 0.3%–1.0% annual risk (68,69). Age at time of exposure appears to be a risk factor in some series, with younger women at higher risk for a contralateral breast cancer. In the study by Boice et al. (70), the RR of contralateral breast cancer was 1.59 in women less than 45 years of age at treatment, whereas women 45 years old and older showed no effect (RR = 1.01). Although the risk of radiation-associated contralateral breast cancers is extremely low, measures to reduce scatter to the opposite breast, such as omission of a medial wedge from tangent fields (71), should be used to reduce contralateral breast dose.

The most recent update of the meta-analysis of RT trials (16) demonstrated a highly statistically significant benefit in breast cancer-specific survival following RT that was largely offset by an increase in vascular deaths. Although the cardiac component of the vascular deaths could not be specifically defined, almost certainly a large percentage of these vascular deaths were cardiac in origin. Previous reports (72–74) have described the excessive total doses and dose/fractions delivered to large volumes of the heart in the earlier trials, which dominate the results of the overview. When Rutqvist et al. (75) reanalyzed the doses received in the Stockholm trial according to the estimated doses to the myocardium, patients who received the highest doses had a statistically significantly increased rate of death caused by ischemic heart disease when compared with surgical control subjects (76). More recent studies (77,78) examining the risk of cardiac events using modern radiation techniques have not found differences by laterality; however, increased follow-up is needed because of the latency of radiation-induced cardiac disease. Højris et al. (79) analyzed the cardiac events in patients randomly assigned in Danish trials 82b and 82c and reported a relative hazard of morbidity from ischemic heart disease of 0.86 for the RT versus no RT patients and a hazard for death from ischemia of 0.84 after 12 years. Therefore, it appears that careful treatment planning and use of low-energy electrons to treat the chest wall and internal mammary lymph nodes have resulted in no increase in cardiac toxicity in the Danish studies at 12 years. Further follow-up will be needed to verify continued constant hazard rates with time.

Despite improvements in RT planning techniques, the potential for additive cardiac toxic effects of RT and systemic therapies mandates careful radiation treatment planning. A study reported by Shapiro et al. (80) demonstrated the potential for cardiac toxicity with high-dose doxorubicin and cardiac irradiation, with an eight- to 10-fold risk of cardiac toxicity at a dose of 450 mg/m² of doxorubicin and RT compared with a negligible risk at a dose of 225 mg/m². With the known cardiac effects of doxorubicin and trastuzumab (currently in trials for adjuvant use) and the radiosensitizing effects of paclitaxel (81–83), RT planning using computed tomography-based systems should be used to minimize cardiac exposure. Funding for continued advances in RT treatment planning should be supported to maximize survival gains.

SUMMARY

Randomized trials have demonstrated that locoregional radiation following mastectomy in patients treated with systemic therapy reduces locoregional recurrence and increases survival.

The majority of patients in these trials were at high risk for LRF. On the basis of the results of these studies, locoregional radiation is recommended to these patients following systemic therapy. The role of locoregional radiotherapy following mastectomy in patients with one to three axillary lymph nodes is currently undefined and is being evaluated in an ongoing randomized trial. Modern techniques should be used to avoid excessive radiation to the heart, lungs, and other normal tissues. Locoregional radiation should be directed to the chest wall, supraclavicular lymph nodes, and axillary apex. The role of IMN irradiation is unclear. Trials are now in progress to evaluate the potential benefit of IMN treatment.

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Side Effects, Quality-of-Life Issues, and Trade-offs: the Patient Perspective

Amy S. Langer

Increasingly effective adjuvant treatments of invasive breast cancer and their widespread use have improved survival rates. Given the timing required by its use, adjuvant therapy requires the patient to absorb complex medical data and make challenging trade-offs shortly after initial diagnosis. However, many women are unprepared or unable to optimize adjuvant treatment decisions while experiencing the shock and dismay that often follow the confirmation of an invasive breast cancer diagnosis. Each woman needs to know the facts and circumstances of her own case and to fully understand the benefits and risks of adjuvant therapy. Only then can she, with her medical team, choose those therapies that will maximize her benefit as a patient and as a survivor in all aspects of her life, over both the short and longer term. To help the patient accomplish these goals, individualized practical knowledge that complements population-based advances in survival is critically needed. Considerable focus, study, and cross-disciplinary collaboration will be required to compile successful, integrated approaches to adjuvant therapy that reflect varying patient contexts and concerns. Other crucial ingredients are the investment of resources in recently established research fields (such as the tracking of psychosocial outcomes and delayed morbidity) and informed guidance from patient advocates. To accelerate patient-centered progress in adjuvant therapy for breast cancer, areas that need attention include targeted public education programs; patient information and informatics; treatment selection and decision-making tools; and interventions and therapies to improve quality of life for patients, survivors, and their families. Underlying all of these efforts should be culturally competent, multigenerational approaches to communicating effectively with diverse patients and family members in multiple clinical and community settings. [J Natl Cancer Inst Monogr 2001;30:125-9]

Over the past decade, surveys have shown that most insured women are receiving recommended regular screening mammograms and clinical examinations in increasing proportions (1). With current compliance rates now approaching 80% in many groups, a resulting national stage shift to earlier breast cancer diagnosis has been observed (2). Building on this positive public health trend, recent advances in the variety and effectiveness of adjuvant treatment options for breast cancer are producing more favorable outcomes for many women with early-stage disease (3). However, considerable effort, application, and discovery lie ahead to extend this progress to women from all backgrounds and in all income levels and groups.

THE 1990s: ENVIRONMENTAL INFLUENCES AND HISTORICAL PERSPECTIVE

Prior to the November 2000 National Institutes of Health Consensus Development Conference: Adjuvant Therapy for

Breast Cancer, the most recent consensus development conference on breast cancer treatment was in 1990. Key elements of the progress made in breast cancer detection and treatment over this 10-year period are useful in understanding the current patient issues in adjuvant treatment that need attention. The June 1990 consensus development conference "Treatment of Early-Stage Breast Cancer" panel established that breast-conservation treatment was preferable to mastectomy for the majority of early-stage patients in the United States (4). This finding, important to clinicians, was of greatest importance to breast cancer patients and survivors, who were collectively on the brink of a transformation in their attitudes, approaches, and reactions to the disease.

Advocacy

The 1990s were the decade of breast cancer advocacy. The grassroots breast cancer patient advocacy movement gained momentum and visibility in the early 1990s, inspired by the earlier accomplishments and advances brought about by organized and unwavering AIDS activists. With breast cancer no longer considered a taboo subject or "the Big C," the topic burst on the public scene through extensive media coverage of the National Breast Cancer Coalition's highly successful "Do The Write Thing" national letter-writing campaign in 1991, which delivered over 600 000 messages to President Bush and members of Congress from every state. The survivors turned activists were articulate and persuasive in demanding expanded breast cancer research efforts, more funding, progressive legislation, and increased involvement of women affected by the disease in key medical and policy decisions (5).

Breast cancer research expenditures at the National Cancer Institute (NCI) rose 64.8% in the period from 1993 to 1998 within a 23.2% overall NCI research budget rise (6,7), largely because of the efforts of this well-organized patient movement. The NCI research budget for other women's reproductive cancers, prostate cancer, lung cancer, and all other major cancer sites and types reported also rose over this period (7). Activists joined medical professionals in addressing quality issues, reimbursement, and entitlement coverage, including the U.S. Food and Drug Administration (FDA) regulation of mammography facilities through the 1994 implementation of the Mammography Quality Standards Act, the growth of the U.S. Centers for Disease Control and Prevention's program to screen low-income women for breast and cervical cancer, and extension of Medicare mammography coverage to annual screening.

Awareness

Public- and private-sector education programs in the 1990s featured the personal stories of celebrities, and well-recognized

Correspondence to: Amy S. Langer, M.B.A., National Alliance of Breast Cancer Organizations, 9 East 37th St., New York, NY 10016 (e-mail: alanger@nabco.org).

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computer products companies with household names launched coordinated marketing efforts to increase national awareness of "good breast health." Public health and media experts joined together in print and broadcast programs to decrease the stigma, myth, and fear frequently associated with the disease. By the late 1990s, the World Wide Web became the newest channel to influence the changing environment of vastly increased breast cancer consumer input. A wide range of information and misinformation—medical and scientific, personal anecdote and commercial perspectives, support and connection, and treatment input—became easy to find and omnipresent, with "24/7" availability and instant delivery at an estimated 17 000 Web sites (Iverson D: personal communication).

Screening Mammography

Some notable shifts in U.S. population and breast cancer patient demographics took place in the years following the 1990 consensus development conference on breast cancer treatment. After several unsuccessful medical and government attempts to arrive at a national consensus on breast cancer screening guidelines, inconsistency and disagreement among experts discouraged education efforts, stalled advances in mammography compliance, and confused women. However, by late in the decade, positive outcomes from maturing worldwide randomized trials and European national screening programs became available, and FDA-regulated mammography quality had begun to improve. After a January 1997 consensus development conference on "Breast Cancer Screening for Women Ages 40–49," most private and public agencies in the United States were able to reach consensus about screening women younger than age 50 years. When revised guidelines were communicated to the public, the majority of leading national breast cancer and cancer agencies and organizations agreed on the need for either annual or "regular" breast cancer screening with mammography starting at age 40 years and regular clinical breast examinations. Screening compliance rates rose in published private and government screening surveys (8,9). In the 1998 release of 1996 data from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER)¹ cancer database, a pronounced shift to earlier-stage breast cancer diagnoses, as well as survival improvements, began to be evident (10). According to SEER, over the period from 1983 to 1987, 53% of new invasive female breast cancer cases were diagnosed at "localized" stages, a figure that rose to 62% by 1996 (2,11). Increased mammography use also resulted in a disproportionate increase in the detection of *in situ* (preinvasive) breast cancers. While the number of new female invasive cases rose about 5% (from 175 000 to 182 800) over the period from 1990 to 2000, the number of new *in situ* cases increased by 184% (9,12).

Broad U.S. population shifts have also contributed their effects. America is "graying," with a large and growing demographic segment of senior women (13), a group at high breast cancer risk because of age. Women currently in their seventies in the United States face up to a one in 25 chance of being diagnosed with breast cancer over the next 10 years (14), yet they frequently misunderstand risk factors—for example, they assume that their daughters are at greater risk than they are themselves (unpublished data, National Alliance of Breast Cancer Organizations/WeightWatchers Survey, 1999). Senior women, who often retain 1960s and 1970s disease associations (of losing both your breast and your life), continue to comply with mam-

mography screening at one of the lowest rates of any U.S. group (15) despite improved Medicare screening coverage.

Demographics also predict a larger number of breast cancer cases in younger women in the United States, although invasive incidence rates for those women under 60 years of age have remained stable (2). Although the average age at diagnosis for breast cancer in the United States is currently about 64 years of age (2) and 56% of diagnoses occur after age 60 years (8), the aging of the post-World War II generation is swelling the ranks of women in their forties and fifties. As a result, with stable breast cancer incidence rates in these age ranges, over the next several years, the absolute number of women in America diagnosed with invasive breast cancer before age 60 years will be the highest in history, merely reflecting the prevalence of this age group in the U.S. population. Younger women—among those most likely to receive screening mammograms—are relatively well-educated, media-savvy consumers often comfortable with technology. They are increasingly health conscious and are insured and in the workforce more often than their mothers were—age-specific behavior observed in most cultural and population groups (8).

Disparities and Stage at Diagnosis

Increased compliance with screening mammography recommendations has resulted in a shift to earlier stage breast cancer diagnosis. However, this shift (and the resulting improvement in survival) has largely been confined to insured and higher income women, for whom mammography is accessible and affordable. Women's health surveys that have historically shown education and income to be strong determinants of health-seeking behavior reflect consistent trends in breast cancer screening, mammography use, stage at diagnosis, and survival.

In breast cancer, differences in income rather than race predict the greatest disparities in access to screening and treatment, leading to differing health outcomes. The NCI's SEER cancer database, the largest source for national breast cancer statistics, reports cancer information by race rather than by income, using white and black as principal categories. However, differences between the SEER data for black and white women do reflect the effect of income disparities to some extent. Invasive female breast cancers reported by SEER as localized—an encouraging 62% of all new SEER cases from 1989 through 1996—are early-stage invasive diagnoses (2). Recently published SEER data on breast cancer stage at diagnosis for black and white women includes 1997 stage 1 diagnoses: the earliest-stage invasive, lymph node-negative cases less than 2 cm in diameter, principally mammographically detected. Illustrating the current disparity in access to care, in 1997, 41% of the SEER breast cancers in white women were diagnosed at stage 1, compared with only 28% in black women (2).

THE 1990s: KEY CLINICAL AND RESEARCH TRENDS

In considering adjuvant therapy from the patient's perspective, there have been several important and influential clinical trends since the 1990 consensus development conference. Advances in primary and localized treatments—especially less radical mastectomy and increasing acceptance of breast-conserving treatment—have overcome certain morbidities and have been responsive to quality-of-life concerns. However, important open questions in primary and local breast cancer therapy, such as the usefulness and appropriate application of postmastectomy radia-

tion therapy, still remain unresolved. Research showing the benefit of adjuvant systemic therapy for early-stage disease was widely reported and well publicized, beginning with a "Clinical Alert" (16) from the NCI in the late 1980s. A series of studies and trial results published through the 1990s reported decreased recurrence and improved survival benefits from adjuvant therapy as new combinations and sequences of agents created more choices and asked patients to evaluate probabilities and statistics and to trade-off benefits and side effects. Among the many open issues in this area that are critical for patients are questions of dose density and dose intensity, hormonal versus cytotoxic agents and their combinations, and the interdigititation of local and systemic approaches.

With expanding adjuvant therapy strategies, approaches, and agents, patients and their families require more information, more complex and diverse input, and effective communication from their medical teams to make informed treatment decisions. However, as more women with breast cancer must make more complicated treatment decisions, the advent of managed care has meant that time for individual patient interaction is shrinking in offices and clinics. In addition to more prepared, communicative, and empowered breast cancer patients being diagnosed in the decade of advocacy, a new focus on "patient-centered" (as opposed to "tumor-centered" or "disease-centered") areas of cancer research has helped medical and oncology professionals to perceive and address this communication challenge. In disciplines formerly overlooked or rejected as "soft science," researchers began to recognize and assess the longer-term physical impact of adjuvant treatments as well as the emotional, sexual, societal, and psychosocial quality-of-life effects of breast cancer. Influential breast cancer advocates have had dual roles, by stimulating this research through increased funding as well as by demanding recognition of and interventions for women's non-medical needs—both during active treatment for breast cancer and into survivorship.

SHORTCOMINGS OF CURRENT APPROACHES TO ADJUVANT THERAPY

Despite its progress and achievements, state-of-the art adjuvant therapy for breast cancer has multiple shortcomings from the patient's perspective, both on the macro level and for the individual patient. In terms of bigger-picture issues, adjuvant therapy is generally recommended by physicians and selected by patients by correlating a woman's tumor characteristics (and, to a lesser extent, personal factors such as her age) with historical trial and research outcomes. Although scientifically based and data driven, this method assumes homogeneity among trial populations and makes broad assumptions about the applicability of trial results to very diverse individual women. The long-term outcomes of population-based treatment studies that opened for enrollment more than 15 years ago—especially since they attract fewer than 5% of U.S. breast cancer patients (17)—may not be a good match with the highly heterogeneous women who are breast cancer patients in the United States today.

A second major drawback of current adjuvant therapy is that it is not sufficiently individualized. Only very rudimentary tumor and patient characteristics are currently used as prognostic or predictive factors to forecast risk of recurrence and treatment effectiveness. As a result, this approach overtreats some women who would remain disease free with lower doses or different

treatments or none at all, exposing them unnecessarily to morbidity, expense, and side effects. At the same time, it undertreats or incorrectly treats women whose breast cancer recurs, exposing them to a high risk of dying of the disease. Women who can be identified in the undertreated or overtreated groups are evident only in retrospect, through later symptoms.

Among other large-scale problems is that the effectiveness of current therapy is limited: It is still not a cure. We cannot predict the impact of short-term or long-term therapeutic side effects or monitor future response to adjuvant therapy while it is ongoing, so we cannot yet change therapeutic courses if a treatment becomes ineffective. Clinical trials that establish future directions for adjuvant therapy take considerable time from design to completion, are expensive, often produce only incremental results, and offer limited provider and patient incentives for enrollment. Few studies and even fewer data exist on patient choice topics (such as treatment decision making, risk assessment, and quality-of-life effects) that could improve women's clinical trial experiences and raise enrollment as a result. Both the medically underserved and women in diverse minority populations—an increasing percentage of breast cancer patients—are not well represented in many trials and too often do not receive state-of-the-art treatment.

For the individual patient, adjuvant therapy is a complicated opportunity, offering the promise of benefits as well as substantial drawbacks. Making informed therapy choices requires immersion in medical information and the consideration of difficult decisions made rapidly after diagnosis, and women can feel unprepared and overwhelmed. Therapy selection is often based on quantitative factors and abstractions, such as percentage lower chance of recurrence, relative risk, and gain in percentage likelihood of surviving 5 years, all ranging from certainty to the unknown. These concepts can be difficult to comprehend and internalize, even under nonstressful conditions, and are presented by medical professionals in the absence of widely published research on accepted, effective communication techniques and tools. In addition, experts have observed limited "numeracy" among adult Americans, who have low levels of facility and comfort with percentages and quantitative factors (Iverson D: personal communication).

More easily understandable by patients, but too often incompletely explained or not communicated well enough by health care professionals, are treatment-related side effects like hair and fertility loss, nausea, hot flushes, and effects on cognition and memory, as well as what can be done to avert, reverse, or ameliorate them. Even less is disclosed, and far too little is understood, about the toxic effects of longer term treatment, such as cardiac damage and secondary cancers, and about an individual woman's safe tolerance level and lifetime capacity for any one powerful chemotherapy drug. As new agents move rapidly to the clinic before their longer term and side effects are well known, "informed consent" becomes a moving target, and approaches not mentioned by the physician cannot be considered by the patient. Too often, women commence adjuvant therapy with inadequate information and preparation for its physical and emotional effects and, frequently, individual responses differ. While in active treatment, over the short term, a patient can experience an emotionally fragile state in which it is difficult to predict and plan; when treatment is complete, patients take on the burden of living with the risk of recurrence. Once breast cancer is diagnosed, there is little emotional relief.

Science is advancing from its current state of knowledge to the certain prevention and cure of breast cancer. In the meantime, if physicians could offer and women could choose from a range of adjuvant therapy options for breast cancer, what would be the ideal characteristics of that therapy? From the patient's perspective, adjuvant breast cancer treatment options should be clinically well understood, selective, tailored, well communicated, and effective. More about each characteristic is explained below.

Clinically well understood means that the patient can obtain both qualitative and quantitative information about the therapeutic option, available to her from multiple accessible and understandable sources that amplify, complement, and confirm what she needs to know. Sources for input would include her physician and medical institution, the peer-reviewed oncology literature, reliable and familiar information sources that she trusts (such as nonprofit and government organizations and agencies), and other women and patients in support groups or other settings. Both qualitative and quantitative information would be available about treatment risks (e.g., morbidity and mortality, their variability and likelihood) and treatment benefits (e.g., recurrence; survival; side effects; and practical, economic, and quality-of-life aspects). The information would address risks and benefits during active therapy, over the short term, over longer term follow-up, and into survivorship. It would embrace all aspects of the woman's personal and family life as well as treatment effects on her life partner, close family members, friends, and coworkers.

Selective means that the treatment is most likely to maximize disease response and prevent recurrence with the fewest, or no, adverse side effects. Its choice would be based on her disease specifics, features and characteristics, her personal health and cancer history, her family health history and genetic profile, and the state of her overall health.

Tailored treatment is individualized treatment, chosen while taking into account the patient's personal priorities, reactions, preferences, and attitudes about risk, side effects, and quality of life. Considerations would include her point of view about symptom trade-offs, reserving future treatment and drug capacity, and her perception about the sequencing of options; her ranking of the importance of side effects, including those that can be modified (and how), and those that cannot; and the meaning to her of various dimensions of quality of life—both in her life at the moment and as she projects her future, both at work and at home. Treatment would consider how and when she most values clinical research and contributing a legacy to future generations, as well as process-oriented aspects of trial participation: consent, randomization, privacy, and monitoring.

Tailored treatment would also take into account the patient's age, stage of life, and personal priorities, including physical—strength and physical requirements at work and at home, capacity for energy and reserves, fertility and sexual issues, and tolerance for hot flushes, flashes, and night sweats; cognitive—how and when she most values stability/transience of mood, acuity, focus, and memory; emotional—issues about depression, anxiety, and body image, including the effect of partnering status and other support systems; and practical—treatment costs and adequacy of reimbursement, her physical mobility and avail-

ability of transportation, obligations of dependents, and requirements for predictability/tolerance for variability.

Well-communicated treatment options are conveyed and discussed using language and decision-making methods that are understandable, meaningful, and successful and that assist and support the individual woman in making informed, confident decisions. Medical providers who are effective communicators can assist the patient in this process by helping her to listen, hear, absorb, and understand the information and choices that are being suggested (*see* Table 1). Although additional tools and techniques are needed, existing effective approaches should be used to put the patient at ease, to establish informative two-way communication, and to both permit and encourage the woman to compare appropriate alternatives side by side in partnership with her health care team (*see* Table 2). Well-communicated treatment discussions empower the patient to take an active role in making decisions that will affect the length and quality of her remaining life. In some cases, a recurrence-free future is beyond the promise of current science and, for these cases above all, well-communicated options include each treatment's limitations

Table 1. Communicating breast cancer adjuvant therapy options: steps in effective patient/provider communication

Help the patient to . . .	By these approaches to communication . . .
Listen	Maintain eye contact, speak respectfully, remember context of high level of fear, anxiety, and unfamiliar medical setting
Hear	Give the discussion sufficient time, speak slowly and clearly, use plain language, and avoid medical jargon; determine if English is not native language (<i>see</i> "Understand" below)
Absorb	Observe cues that the concepts are being received, use active listening techniques to repeat/rephrase, and clarify what patient says
Understand	Increase comprehension and retention of information so it can be retold later to family/friends through encouraging note taking, offering handouts and take-home material to reinforce, use repetition, and suggest that the patient bring a companion to important discussions
Compare	Encourage the patient to compare choices and share options and discussions with others, including informed women (such as in a support group), noting additional questions to be discussed
Consider	Early-stage breast cancer is not an emergency, and careful consideration of treatment options and additional input from a variety of sources are components of an informed treatment decision
Decide	Within 4–6 weeks from primary treatment, reach a well-considered adjuvant therapy decision, and begin treatment

Table 2. Communicating breast cancer adjuvant therapy options: recommended approaches that encourage and empower the patient

Encourage the patient to . . .	With this result . . .
Relate her breast cancer story	Establish who she is as an individual
Explain her life	Offer useful context
Reveal her decision-making style	Provide insight into treatment trade-offs
Express fear and anger	Reassure about what is normal, appropriate
Challenge and question	Engage actively, feel more in control
Explain daily needs	Choose practical treatment
Find resources and information	Participate actively, understand more
Seek out survivors	Gain support, knowledge, and inspiration
Feel empowered	A full partner makes better, more informed choices

and an unwavering commitment to tell the truth as the medical team knows it. For many patients, knowing what to expect, even if it is not the best outcome, can reduce anxiety.

Effective treatment is treatment that works—in the adjuvant setting, treatment that delays or prevents the recurrence of breast cancer. However, to be effective for an individual woman, even therapy with this successful end result must have an acceptable price in its effects on her life. And for patients with higher risk disease where prevention of recurrence may not be a realistic outcome, the definition of effective therapy will vary. Ultimately, the choice is the patient's, once the medical professional has met the obligation of clearly communicating the most appropriate, tailored, and selective adjuvant treatment options available. In providing effective choices, the professional has the obligation to take direction from the patient, to advise of and explain clinical research options, to update and reassess when new information becomes available, and to consider complementary and alternative therapies. Effective therapy can include pausing to rethink and reconsider and always includes the prompt, private, and sensitive delivery of bad news.

ADJUVANT THERAPY FOR OUR DAUGHTERS

What adjuvant therapy for breast cancer does the future hold? Not so far away, for our daughters or for their daughters, may be universal breast cancer prevention, perhaps as a vaccination. An educated and proactive public could obtain risk profiles early through simple tests when all of the causes of breast cancer are known. Adjuvant therapy may no longer be necessary when better screening and imaging techniques make truly early "early detection" possible, when a breast cancer is still a mere clump of cells. Then, perhaps the errant cells could be reprogrammed or minimally invasive ablation could be possible, similar to how a small skin cancer is treated today—on a woman's lunch hour. Successful tumor-specific strategies coupled with patient-specific approaches would be independent of today's seemingly insurmountable time, reimbursement, and access problems. Although these may sound like distant possibilities, the combined power of advocacy, passion, science, and research have brought us far closer than we were just 10 years ago. Progress in adjuvant breast cancer therapy advances us further on the path to the future, to healing the immense pain and loss that breast cancer causes, and to eliminating its current, vast threat to America's women and their families.

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NOTES

¹*Editor's note:* SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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Impact of Tamoxifen Adjuvant Therapy on Symptoms, Functioning, and Quality of Life

Patricia A. Ganz

This article reviews the symptoms and everyday problems associated with tamoxifen adjuvant therapy and their impact on patients' quality of life. In addition, the purported toxic effects of tamoxifen therapy (e.g., premature menopause, weight gain, and depression) are discussed, and data are presented that refute claims of the toxicity of tamoxifen therapy. From randomized controlled trials of adjuvant therapy, we know that tamoxifen therapy increases the rate of hot flashes, night sweats, and vaginal discharge; however, in observational studies these symptoms do not have a statistically significant impact on patients' quality of life as measured by standardized, self-report questionnaires. The Breast Cancer Prevention Trial found no evidence of excessive rates of depression or clinically significant differences in sexual functioning between women receiving placebo and those receiving tamoxifen therapy. Although several serious medical risks from tamoxifen therapy exist (e.g., uterine cancer, blood clots, stroke, and cataracts), there are additional benefits from tamoxifen therapy in addition to an increase in disease-free survival rates and overall survival rates, including a decrease in contralateral breast cancer and fractures. Ultimately, the decision to receive tamoxifen therapy is a personal choice for each woman to make on the basis of the evidence of tamoxifen therapy's benefits and risks, along with her own motivation to receive therapy. When the benefits of such therapy are small, some women may choose to avoid treatment, but others may wish to try therapy to determine whether possible side effects are relevant. For women in whom the absolute survival benefits are large, there may be less difficulty in making this decision. [J Natl Cancer Inst Monogr No. 30:2001;130-4]

Tamoxifen therapy has been an integral part of systemic adjuvant therapy since the early 1980s. This therapy was first used primarily in postmenopausal, lymph node-positive patients and subsequently in both premenopausal and postmenopausal patients with hormone receptor-positive, lymph node-negative tumors. More recently, tamoxifen therapy has been demonstrated to benefit women with noninvasive breast cancer and women at high risk for breast cancer. While the acute toxic effects of tamoxifen therapy are mild compared with combination chemotherapeutic regimens, concerns related to the side effects of this therapy have become more prominent with the increasing use of this agent in women with very early-stage disease [or women who are high risk (1) only] where the absolute gains in survival are modest. If symptoms associated with a treatment are nearly universal, and the absolute benefit of the therapy is small, then one can begin to question the personal costs of such therapy. This is the current dilemma for women with very early-stage breast cancer who must decide whether or not to receive tamoxifen adjuvant therapy.

In this article, I will review the everyday symptoms and qual-

ity-of-life concerns associated with tamoxifen adjuvant therapy, the impact of tamoxifen on sexual functioning, the serious medical risks associated with the drug, and important considerations related to treatment decision making about the use of tamoxifen therapy in the adjuvant setting. Ultimately, the decision to receive this therapy will rest with the patient; however, physicians are obligated to understand the benefits and risks of tamoxifen adjuvant therapy and to guide their patients in the decision-making process.

WHAT ARE THE EVERYDAY SYMPTOMS AND QUALITY-OF-LIFE CONCERNS ASSOCIATED WITH TAMOXIFEN ADJUVANT THERAPY?

After receiving a diagnosis of breast cancer and undergoing one or more of the various treatments prescribed to treat the disease (i.e., surgery, radiation therapy and adjuvant chemotherapy, or hormonal therapy), women have offered anecdotal reports of a range of symptoms that have been attributed to tamoxifen therapy, including weight gain, hair loss, joint pain, fatigue, depression, vaginal dryness, vasomotor symptoms (i.e., hot flashes and sweats), and diminished sexual functioning. Many of these symptoms are directly related to chemotherapy-induced menopause or to withdrawal of hormone replacement therapy; however, patients often attribute these symptoms to tamoxifen therapy, which is usually initiated subsequent to chemotherapy and hormone withdrawal. Furthermore, clinicians tend to remember very troubled patients, for whom the use of tamoxifen therapy seems to be associated with severe symptoms that have a major impact on quality of life, even though large numbers of their female patients tolerate this medication well and do not report changes in quality of life. In fact, many of the symptoms that have been attributed to tamoxifen therapy, such as vaginal dryness and weight gain, are now well-known to be concomitants of normal aging in women as they enter menopause (2,3).

It is important to move from anecdote to evidence as we consider the potential risks and side effects of tamoxifen adjuvant therapy. There are several good sources of data from descriptive longitudinal studies, cross-sectional comparison studies, and randomized controlled trials that use a placebo. Evidence from these studies will be used to discredit several myths about tamoxifen therapy as well as to document those symptoms that are increased in frequency as a result of this

Affiliations of author: University of California-Los Angeles Schools of Medicine and Public Health; Jonsson Comprehensive Cancer Center at University of California-Los Angeles.

Correspondence to: Patricia A. Ganz, M.D., Division of Cancer Prevention and Control Research, Jonsson Comprehensive Cancer Center at University of California-Los Angeles, Box 956900, Rm. A2-125 CHS, Los Angeles, CA 90095-6900 (e-mail: pganz@ucla.edu).

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therapy. Where available, data that examine the impact of symptoms on quality of life will also be described.

Does Adjuvant Tamoxifen Therapy Induce Premature Menopause?

There has been considerable interest in the risk of adjuvant chemotherapy inducing premature menopause (4), with limited prospective data available on this issue. Goodwin et al. (5) prospectively studied an inception cohort of 183 premenopausal women with locoregional breast cancer who received several forms of adjuvant therapy or no adjuvant treatment and followed up their status for 1 year to examine the predictors of the onset of menopause. No treatment was received by 29% of the sample; 12% received tamoxifen therapy alone; 45.3% received either cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) or cyclophosphamide, epirubicin, and 5-fluorouracil (CEF); and 13.6% received either CMF or CEF followed by tamoxifen therapy. The multivariate model in this study included the following predictors: age, tumor size, lymph node status, chemotherapy, hormone therapy, and all relevant interaction terms. The final model found that age ($P<.00001$), chemotherapy (either CMF or CEF; $P<.0001$), and tamoxifen therapy ($P = .034$) were each statistically significantly and independently associated with the onset of menopause. The use of tamoxifen therapy in addition to either type of chemotherapy resulted in a small but statistically significant increase in the risk of menopause (4).

Goodwin et al. then went on to develop a model to predict the probability of the development of menopause according to age at diagnosis and type of adjuvant therapy (Fig. 1). The 95% confidence intervals for the chemotherapy and combined therapy curves overlap, as do the curves for tamoxifen therapy and no therapy. What this probability model demonstrates (Fig. 1) is that beyond the age of 35 years, the risk of menopause is statistically significantly increased when chemotherapy is used as opposed to tamoxifen therapy only or to no adjuvant therapy (5). These data provide important information for women who may be concerned about the risk of the onset of menopause associated with adjuvant therapy. While there may be some slight increased risk of premature menopause from tamoxifen therapy in the oldest group of premenopausal breast cancer patients, for women younger than 45 years of age, this is not a substantial

risk. Even in older menstruating women, this model suggests that the incremental increased risk of menopause from tamoxifen therapy is only about 10% greater than in those women who receive no therapy (5).

Does Adjuvant Tamoxifen Therapy Cause Weight Gain?

Clinician and patient concern about weight gain after adjuvant chemotherapy have been noted for about two decades (6), and the same concern has been raised anecdotally about tamoxifen therapy, which has only recently been used more extensively in the adjuvant setting. Using a large inception cohort of 535 women with newly diagnosed locoregional breast cancer, Goodwin et al. (7) prospectively examined the question of weight gain after breast cancer. The mean age of the women in this study was 50.3 years, and 57% were premenopausal. The sample included patients with lymph node-negative and lymph node-positive tumors, as well as patients receiving no therapy, adjuvant tamoxifen therapy, or adjuvant chemotherapy. During 1 year of follow-up, 84.1% of the patients gained weight. In a multivariate analysis, the onset of menopause and the administration of chemotherapy were independent predictors of weight gain (all $P\leq .05$). Tamoxifen adjuvant therapy was not associated with an increased risk of weight gain (7).

Does Adjuvant Tamoxifen Therapy Contribute to Increased Symptoms or Diminished Quality of Life?

This question can be answered with data obtained in randomized trials as well as from a cross-sectional study of a large sample of breast cancer survivors. There are two randomized, placebo-controlled trials that have evaluated the toxicity of adjuvant tamoxifen therapy. In the Wisconsin Tamoxifen Trial conducted by Love et al. (8), 140 postmenopausal, lymph node-negative patients were randomly assigned to receive tamoxifen therapy or a placebo. With the use of an interviewer-administered questionnaire, patients were asked to evaluate their anxiety, a range of symptoms they had experienced, the overall toxicity of the therapy they had received, and their quality of life. Follow-up occurred during a 24-month period of time. Key findings from this study include an increase in hot flashes reported by women receiving tamoxifen therapy (67.2% versus 45.4% at 6 months; $P<.01$), with severe hot flashes reported by 20.3% of women receiving tamoxifen therapy versus 7.6% in those on placebo assessed at 6 months ($P<.04$), and more frequent occurrence of gynecologic symptoms (i.e., bleeding, irritation, or vaginal discharge) in those receiving tamoxifen therapy (29.7% versus 15.1% at 6 months; $P<.05$), and these were predominantly mild in severity (8). There were no differences between the two groups in reports of the symptoms of nausea, fatigue, bone pain, joint pain, racing heart, vomiting, depression, sweaty hands, irritability, difficulty sleeping, or gastrointestinal distress (8). Finally, there was no adverse effect on quality of life as measured by nonstandardized questionnaires.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 trial (9) in lymph node-negative, estrogen receptor-positive premenopausal and postmenopausal women provides a much larger double-blind, placebo-controlled trial sample for the consideration of this question (>1400 women were included in each treatment arm). However, no self-report data on symptoms or quality of life were obtained in this study. Nevertheless, detailed toxicity evaluation from the B-14 trial demonstrated a pattern of symptoms very similar to those found in the

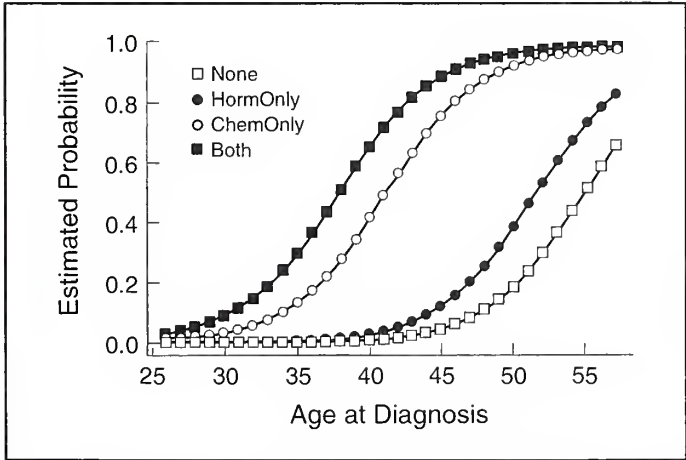


Fig. 1. Model of the estimated probability of developing menopause in the first year after being given a diagnosis of breast cancer according to type of adjuvant therapy received. Adapted with permission from (4).

Wisconsin Tamoxifen Trial (8). Over the course of 5 years of therapy in the NSABP B-14 trial (9), 64.1% of tamoxifen-treated patients reported hot flashes compared with 47.7% of placebo patients. Vaginal discharge was noted in 29.7% of tamoxifen-treated patients compared with 15.2% of placebo patients. There were no significant differences in reports of weight gain, weight loss, fluid retention, nausea and vomiting, or diarrhea. Protocol therapy was discontinued in an equal number of patients assigned to placebo and tamoxifen therapy; however, approximately 50% more patient withdrawals were attributed to treatment toxicity in the tamoxifen arm of the study (9).

In a recent cross-sectional study of symptoms and quality of life in breast cancer survivors an average of 3 years after breast cancer diagnosis, Ganz et al. (10) used state-of-the-art, self-report measures to evaluate these patients according to the type of adjuvant therapy that they received. Hot flashes and night sweats were reported statistically significantly more often by survivors who received adjuvant therapy (i.e., tamoxifen therapy, chemotherapy alone, or chemotherapy plus tamoxifen therapy) than by survivors who had not received any adjuvant therapy ($P < .0001$), with rates of hot flashes in the tamoxifen-treated patients that were comparable to those noted in the placebo-controlled trials described earlier (8,9) (Fig. 2). The frequency of vaginal discharge was also increased among those survivors receiving adjuvant therapy ($P < .0001$), with tamoxifen therapy making an important contribution to this increased rate of symptomatology (Fig. 2). Other symptoms (i.e., weight gain, forgetfulness, vaginal dryness, pain with intercourse, and difficulty concentrating) were not statistically significantly increased among those who had received tamoxifen adjuvant therapy (10). It is important that no statistically significant differences in quality of life or depressed mood could be attributed to the use of adjuvant tamoxifen therapy, in spite of statistically significant increases in vasomotor and vaginal symptoms (10).

The largest and most comprehensive assessment of symptoms and quality of life related to tamoxifen therapy comes from the recently completed Breast Cancer Prevention Trial (BCPT), a randomized, double-blind, placebo-controlled trial that included more than 13 000 healthy, high-risk women (1,11). The BCPT

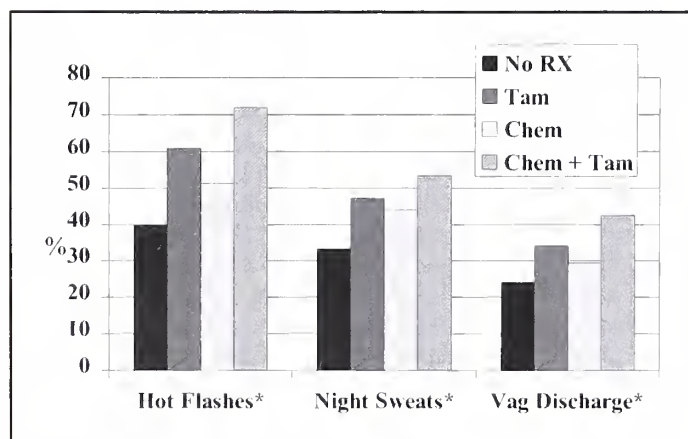


Fig. 2. Percentage of breast cancer survivors reporting symptoms according to adjuvant therapy status. Frequencies presented are unadjusted for age and time since diagnosis, although statistical comparisons are adjusted for these covariates with $P < .0001$. No RX = no adjuvant therapy, $n = 265$; Tam = tamoxifen adjuvant therapy, $n = 356$; Chem = adjuvant chemotherapy, $n = 180$; Tam+Chem = adjuvant chemotherapy and tamoxifen, $n = 295$. Data for this figure are from (10).

used a battery of 104 items taken from standardized, self-report measures of quality of life, depressed mood, everyday problems (including menopausal symptoms), and sexual functioning (12). All participants were assessed before random assignment and at each follow-up visit (11,12). In a report from the first 11 064 women who entered the trial and whose status had been followed up for 36 months, Day et al. (11) could not identify detrimental effects on quality of life or mood from tamoxifen therapy, although vasomotor symptoms and vaginal discharge were statistically significantly increased by the tamoxifen treatment. The side-effect profile of tamoxifen therapy varied somewhat across age groups, with hot flashes being most commonly reported in women in the 50–59 year age group (11).

EFFECT OF TAMOXIFEN ON SEXUAL FUNCTIONING

Research on healthy women and on women with breast cancer demonstrates an age-related decline in sexual functioning (13–15). In the BCPT (11), rates of sexual activity with a partner did not differ by tamoxifen therapy or placebo status, although a subtle decline in sexual activity was noted for both groups across the first 3 years of the randomized trial. However, tamoxifen-treated participants reported slightly increased rates of problems in sexual arousal and difficulty having orgasm (11). In cross-sectional studies by Ganz et al. (10,15) and by Meyerowitz et al. (16) of breast survivors, no statistically significant differences in sexual health and functioning were found in breast cancer survivors compared with healthy postmenopausal women. Furthermore, in a detailed study (17) of the predictors of sexual health after breast cancer, chemotherapy treatment was the only statistically significant treatment-related variable predicting sexual dysfunction, and it was associated with a greater risk of vaginal dryness, a symptom that is usually not related to tamoxifen therapy.

SERIOUS MEDICAL RISKS OF TAMOXIFEN THERAPY

In considering whether or not to take adjuvant tamoxifen therapy, many women with small tumors in the breast weigh heavily the other potential adverse consequences of tamoxifen therapy, such as strokes, blood clots, cataracts, and endometrial cancer (1,18–21). Although the relative reduction in risk of breast cancer systemic recurrence is uniform across all stages of the disease, the absolute benefit decreases relative to disease burden (Fig. 3). As suggested by the examples in Fig. 3, a woman with a very limited tumor burden (e.g., patient A with a 1-cm tumor, negative lymph nodes, and positive hormone receptors) will benefit from tamoxifen adjuvant therapy independent of her age, but she will have a variable risk of side effects (serious medical events or symptoms) based on her age. For a 43-year-old woman with a disease burden equivalent to patient A, the benefits of tamoxifen therapy in terms of decreased recurrence, overall survival, and breast cancer risk reduction in the contralateral breast will likely outweigh the potential for side effects. However, a 75-year-old woman with the same degree of tumor burden may choose to avoid tamoxifen adjuvant therapy because of her higher risk for adverse medical events or side effects. In contrast, all women with a disease burden equivalent to patient C, independent of age, will perceive an increased benefit from tamoxifen adjuvant therapy in spite of variable side-effect profiles.

The competing adverse risks and side effects of tamoxifen adjuvant therapy must be balanced against the potential gains in

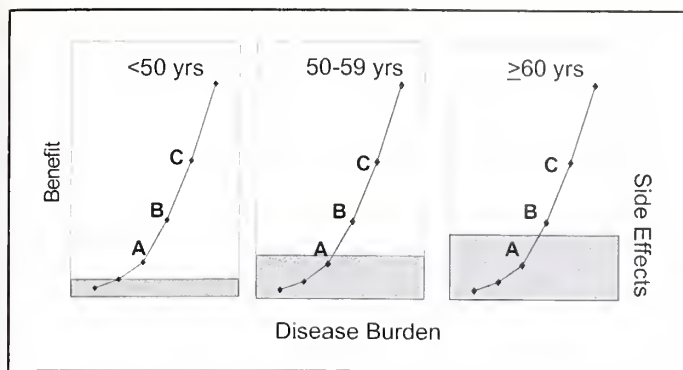


Fig. 3. Relationship among age, disease burden, benefits, and side effects from tamoxifen adjuvant therapy. For patients with disease burden A, B, and C, the absolute benefits of tamoxifen adjuvant therapy are equivalent independent of age group. Only the side effect and adverse event profiles vary by age, with the oldest age group having the greatest risk of serious adverse medical events.

terms of prevention of local and systemic breast cancer recurrence, improved overall survival, and reduction in the risk of contralateral breast cancer. The competing medical risks of tamoxifen therapy are strongly associated with advancing age, other health conditions, and the presence or absence of a uterus. Data obtained from the BCPT (11) on the relative risk and absolute frequency of these adverse events should be used in discussions with women about the risks and benefits of tamoxifen adjuvant therapy. The weights or values each woman will place on a specific adverse risk are very personal and will relate to her perception of her own increased risk for breast cancer recurrence (21).

WEIGHING THE RISKS AND BENEFITS: A PERSONAL CHOICE

The presentation of information to patients about the benefits and risks of tamoxifen adjuvant therapy is complex. Although the relative benefits of this therapy are equally distributed across all ages and tumor stages, the absolute benefits gained for an individual woman are strongly influenced by her disease burden. The question, simply stated, is: How much absolute benefit is required before one should consider taking adjuvant therapy? From a societal perspective, we may ask: How many women must benefit from a treatment before a recommendation can be made that all such women at risk should take the treatment? Furthermore, is survival the only meaningful end point, or is disease-free survival more important for some women? In addition, we must ask about the burden to the patient of taking a medication daily for 5 years that may cause troublesome vasomotor symptoms or vaginal discharge. Does the patient see enough value in increased survival, disease-free survival, or the prevention of a second cancer to offset these symptoms? Finally, does tamoxifen adjuvant therapy also provide some reassurance and sense of protection for women who feel psychologically vulnerable as a result of having been diagnosed with cancer?

Ultimately, the individual woman's perception of benefits and risks, as well as her personal motivation to do something to combat the disease, are the strongest influences in the decision-making process. However, it is important to realize that this treatment can always be discontinued if the woman determines that she has made the wrong decision. In essence, the use of tamoxifen adjuvant therapy in each woman is an "n of 1" trial,

where we will never know with certainty whether the absence of recurrence is the result of this therapy. With careful clinical monitoring, we can assess the actual likelihood of side effects in the individual woman and can either continue or discontinue therapy based on the patient's preference after a trial of the medication.

The challenge to the physician prescribing tamoxifen adjuvant therapy is to understand fully the magnitude of benefits, risks, and side effects of this treatment and to be able to communicate this information effectively to the patient. It is critical to respect the woman's personal preferences and choices. Furthermore, physicians should be gracious about discontinuing this therapy if the patient believes the side effects outweigh the benefits after she has undergone a therapeutic trial of tamoxifen.

CONCLUSIONS

The major symptoms attributable to tamoxifen therapy experienced by women taking this form of adjuvant therapy are hot flashes, sweats, and vaginal discharge. Other common symptoms associated with aging and menopause, such as joint pain, weight gain, changes in mood, and difficulty concentrating, cannot be directly ascribed to the use of tamoxifen therapy but are more likely the result of estrogen deficiency associated with menopause. It is important that tamoxifen adjuvant therapy does not appear to statistically significantly increase the risk of menopause onset in premenopausal women (4). There is no evidence to support poorer quality of life or an increased risk of depression in women who receive tamoxifen as adjuvant therapy after breast cancer (11). These conclusions are derived, however, from the averaging of data from many women, and some individuals within these groups may have adverse experiences. Sexual functioning after breast cancer is adversely affected by the presence of vaginal dryness, which is not a specific side effect of tamoxifen therapy but is probably the result of age-related estrogen deficiency and adjuvant chemotherapy treatment (15,17). Although serious adverse medical events are rare, women are often concerned about these risks of tamoxifen therapy (e.g., blood clots, strokes, and endometrial cancer). Each woman must evaluate all of the risks and benefits of tamoxifen adjuvant therapy when embarking on such treatment. In turn, the woman's physician must guide her in this exercise by providing her with accurate and comprehensive information about her personal situation as to benefits and risks of therapy.

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NOTE

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Side Effects of Chemotherapy and Combined Chemohormonal Therapy in Women With Early-Stage Breast Cancer

Ann H. Partridge, Harold J. Burstein, Eric P. Winer

The decision to receive chemotherapy or chemohormonal therapy involves careful consideration of both the potential benefits and possible risks of therapy. There are substantial short- and long-term side effects from chemotherapy. By convention, short-term side effects include those toxic effects encountered during chemotherapy, while long-term side effects include later complications of treatment arising after the conclusion of adjuvant chemotherapy. These side effects vary, depending on the specific agents used in the adjuvant regimen as well as on the dose used and the duration of treatment. There is also considerable variability in side effect profile across individuals. This review will focus on the short- and long-term toxicity seen with the most commonly used adjuvant chemotherapy and chemohormonal therapy regimens. [*J Natl Cancer Inst Monogr* 2001;30:135–42]

The role of adjuvant systemic therapy has been studied extensively in women with early-stage breast cancer. Chemotherapy and chemohormonal therapy improve disease-free and overall survival in women with operable breast cancer (1). The absolute benefits of adjuvant chemotherapy vary depending on the treatment regimen, the characteristics of the tumor (e.g., hormone receptor status), the medical and demographic characteristics of the woman (e.g., comorbid conditions and age), and the absolute risk of disease recurrence. In women with a relatively high risk of disease recurrence, the improvement in disease-free and overall survival associated with adjuvant chemotherapy can be quite substantial. In contrast, in women with small tumors and/or negative lymph nodes, the absolute benefits of treatment may be quite small. Decision making about adjuvant therapy—particularly adjuvant chemotherapy—can be complex. Women and their physicians must consider the potential benefits of treatment as well as the possible risks and anticipated side effects.

Side effects from chemotherapy can be divided into short-term effects and long-term effects. Table 1 lists short-term and long-term effects of adjuvant chemotherapy. Short-term effects typically occur during the course of treatment and generally resolve within months of the completion of therapy. In contrast, long-term effects can have a later onset and sustained impact—often lasting for many years. In the case of some of the rare long-term effects, many years may elapse before any symptoms develop.

SHORT-TERM SIDE EFFECTS

The most frequently encountered short-term side effects seen with standard adjuvant chemotherapy regimens and their relative frequency and severity are listed in Table 2. Fatigue, which is listed as a short-term effect, has been recognized in recent years as a common side effect of cancer chemotherapy (2–7). The

assessment of fatigue with standard toxicity grading scales has probably underestimated the prevalence of this problem, and there are few studies of women receiving adjuvant chemotherapy that have detailed self-reports of fatigue. For this reason, it is particularly difficult to determine the prevalence, severity, and duration of fatigue in women receiving adjuvant chemotherapy. There is evidence that some patients cite difficulties with fatigue for months and even years after adjuvant chemotherapy (7,8), but it is not known to what extent such findings differ from age-matched control subjects. Because of the presumed underreporting of fatigue in many studies, it is not possible to assess with confidence the prevalence and severity of fatigue associated with different adjuvant regimens.

Treatment-related side effects are often gauged by standardized criteria from the National Cancer Institute. In Table 2, we have characterized the frequency and usual severity of the most common short-term side effects using the reported toxic effects in 12 adjuvant trials conducted in the late 1980s and 1990s (9–21). We have focused on recent trials that have used many of the modern supportive care measures that are currently available; however, some of these trials were conducted before the availability of the serotonin antagonists for the prevention and treatment of emesis. Therefore, reported rates of nausea and vomiting may be somewhat higher than would be seen today. Side effects characterized as mild correspond to reported grade 1 or 2 toxic effects, whereas those characterized as moderate and severe correspond to grade 2 or 3 and grade 3 or 4 toxic effects, respectively. We characterized the frequency of side effects as follows: fewer than 1%, almost never; 1%–5%, rare; 6%–20%, uncommon; 21%–50%, common; 50%–95%, frequent; and greater than 95%, almost always. Similar regimens administered in different trials and by different investigators (9–21) had remarkably similar side effect profiles. It should be noted that, although neuropathy is listed as a short-term side effect, the extent to which this persists over time has not been well characterized.

In general, the non-anthracycline-containing regimens are associated with fewer grade 3–4 short-term toxic effects than are anthracycline-based regimens. Neuropathy is rarely seen with either the combination chemotherapy of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) or the methotrexate and 5-fluorouracil (MF) regimens. In contrast, emesis (i.e., nausea and/or vomiting), alopecia, and myelosuppression (principally neutropenia) are seen commonly to very commonly with the CMF regimen. Mucositis is seen less frequently with intrave-

Affiliations of authors: Dana-Farber Cancer Institute, Brigham and Women's Hospital, Boston, MA.

Correspondence to: Eric P. Winer, M.D., Breast Oncology Center, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115 (e-mail: ewiner@partners.org).

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Table 1. Side effects of chemotherapy

Short-term effects	Long-term effects
Emesis	Premature menopause/infertility
Nausea	Weight gain
Stomatitis	Cardiac dysfunction
Alopecia	Leukemia/MDS*
Myelosuppression	Cognitive dysfunction†
Thromboembolism	
Myalgias	
Neuropathy‡	
Fatigue‡	

*MDS = myelodysplastic syndrome.

†Possible long-term effect; studies are preliminary in nature.

‡May be both short-term and long-term effect.

nous CMF, compared with oral CMF. Despite the frequency of these side effects, they are often of either mild or moderate severity. Complete alopecia can be seen with these regimens, but when alopecia occurs with CMF, it is frequently partial. Because cyclophosphamide is administered orally for a total of 84 days in the classic oral CMF regimen, nausea with this regimen is sometimes more persistent than with other programs. The MF with leucovorin regimen, used in National Surgical Adjuvant Breast and Bowel Project (NSABP) protocols B-14, B-19, and B-20 (11,13,22), is generally associated with even fewer grade 3–4 short-term toxic effects than classic cyclophosphamide-containing regimens. Because of concern that the MF with leucovorin regimen is inferior to CMF (11), it is not used frequently, although when it is used in combination with tamoxifen, the benefits of CMF and MF appear to be similar (13).

Short-term side effects with the anthracycline-based regimens (15,17,19,20) tend to be more frequent and more severe than those with non-anthracycline-containing treatment. Emesis and myelosuppression are very common with all of these regimens and can be severe in nature. Complete alopecia is seen with almost all anthracycline-based regimens. Mucositis appears to

be more common with the 5-fluorouracil-containing regimens, such as combination chemotherapy with cyclophosphamide, doxorubicin (Adriamycin), and 5-fluorouracil (CAF) or 5-fluorouracil, doxorubicin, and cyclophosphamide (FAC), as opposed to doxorubicin and cyclophosphamide (AC). When paclitaxel is used as part of a sequential regimen, neuropathy and myalgias can be seen occasionally, although symptoms are generally mild. Of note, when higher doses of paclitaxel (i.e., 225 mg/m²) were used, as in NSABP B-28, the neuromuscular toxicity was more frequent and may have been more severe (23) than with lower doses (i.e., 175 mg/m²), as those used in Cancer and Leukemia Group B (CALGB) 9344.

An increased risk of thrombosis has been reported in several trials of adjuvant therapy. The risk of thrombosis appears to occur during active treatment and to abate over time. In a trial comparing shorter and longer chemotherapy regimens, Levine et al. (24) reported an increased risk of thrombosis on both arms, but only during the period of active treatment. Women on the shorter-duration chemotherapy arm stopped having thrombotic episodes when chemotherapy was stopped, whereas women in the longer arm continued to have thrombotic events for the full duration of their treatment. There is evidence that the use of concurrent chemohormonal therapy results in a higher rate of thromboembolic complications than does the use of tamoxifen alone (13,25–27). In NSABP B-20 (13), in which women were randomly assigned to receive tamoxifen alone or administered concurrently with either MF or CMF, the incidence of thrombosis was 1.9% in the tamoxifen-treated group, compared with 6.5% and 7.5% in the patients treated with tamoxifen plus MF and tamoxifen plus CMF, respectively. In a Canadian trial comparing tamoxifen alone with chemotherapy plus tamoxifen, the incidence of thrombosis was 2.6% on the tamoxifen-alone arm and 13.6% in the CMF plus tamoxifen arm ($P < .0001$) (27). The use of concurrent chemohormonal therapy may also be associated with a higher rate of thrombosis than chemotherapy alone (26). Given the greater risk of thrombosis associated with

Table 2. Frequency and usual severity of short-term side effects associated with adjuvant breast cancer chemotherapy regimens*

	Nausea	Vomiting	Diarrhea	Stomatitis	Alopecia	Neutropenia	Febrile neutropenia or infection	Thrombocytopenia	Neuropathy	Myalgias
Toxicity regimen										
CMF (oral cyclophosphamide)	Frequent, +/++	Common, +	Common, +	Common, +	Frequent, partial-total	Frequent, ++/+++	Rare	Frequent, +	Almost never†	Almost never†
CMF (all intravenous)	Common, +/++	Frequent, +	Common, +	Uncommon, +	Frequent, partial-total	Frequent, ++/+++	Rare	Uncommon, +	Almost never†	Almost never†
MF	Common, +	Common, +	Common, +/++	Uncommon, +	Uncommon, minimal	Rare, +	Almost never	Almost never†	Almost never†	Almost never†
AC	Frequent, +/++	Common, +/++	Uncommon, +	Common, +/++	Almost always, total	Frequent, ++/+++	Rare	Uncommon, +	Almost never†	Almost never†
AC-tamoxifen (tamoxifen only)	Rare, +	Rare, +	Rare, +	Rare, +	Almost always, total	Common, +	Rare	Almost never†	Uncommon, +/++	Common, +/++
CEF/FAC (oral cyclophosphamide)	Frequent, ++/+++	Frequent, +/++	Common, +/++	Frequent, ++/+++	Almost always, total	Almost always, +++	Common	Frequent, +/++	Uncommon, +	Uncommon, +
CAF/FAC/FEC 100 (all intravenous)	Common, ++/+++	Common, +/++	Common, +/++	Frequent, +/++	Almost always, total	Frequent, +++	Common	Frequent, +/++	Uncommon, +	Uncommon, +

*Frequency: almost never = less than 1%; rare = 1%–5%; uncommon = 6%–20%; common = 21%–50%; frequent = 51%–95%; almost always = more than 95%. Severity (for all toxic effects excluding alopecia): + = mild; ++ = moderate; +++ = severe. CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; AC = doxorubicin and cyclophosphamide; CAF = cyclophosphamide, doxorubicin, and 5-fluorouracil; FEC = 5-fluorouracil, epirubicin, and cyclophosphamide; MF = methotrexate and 5-fluorouracil; CEF = cyclophosphamide, epirubicin, and 5-fluorouracil; FAC = 5-fluorouracil, doxorubicin, and cyclophosphamide.

†Not recorded in trials (9–21).

tamoxifen in women over 50 years (28,29), combination therapy may be particularly problematic in older women (27). A U.S. Intergroup Trial (30) compared CAF followed by tamoxifen with CAF and concurrent tamoxifen in postmenopausal women. To date, no results comparing these two arms of the study have been reported concerning either efficacy or thrombosis risk. Because of concern about the increased risk of thrombosis, many physicians choose not to administer chemotherapy and tamoxifen concurrently outside of a clinical trial.

Because short-term side effects typically resolve with therapy, the duration of treatment has a major impact on the total side effect burden that a woman may experience. Most treatment regimens are approximately 4–6 months in duration. The AC regimen, however, is substantially shorter and is completed in 12 weeks. The last dose of AC is actually administered 9 weeks after the first dose (9), and, as a result, the duration of short-term side effects is reduced. In a randomized trial comparing AC with 6 months of CMF, investigators from the NSABP concluded that the shorter regimen was associated with a lower total side effect burden (9). The perception that AC is a relatively well tolerated regimen has led to its widespread use over the past decade.

The impact of adjuvant chemotherapy on quality of life has been evaluated in several studies. The International Breast Cancer Study Group randomly assigned patients to either three or six cycles of chemotherapy and demonstrated a more rapid improvement in quality of life with the shorter treatment regimen compared with the longer treatment regimen (31). Other investigators have demonstrated that quality of life improves rapidly with the completion of therapy. Levine et al. (15) showed that quality of life actually improved throughout the course of adjuvant therapy, suggesting some measure of psychological and physical adaptation to a new diagnosis of breast cancer, surgery, and ongoing chemotherapy. Since no studies have measured quality of life before the diagnosis of breast cancer, it is unknown when or if quality of life following adjuvant therapy returns to the prediagnosis baseline. Research in breast cancer survivors suggests that the majority of women diagnosed with early-stage breast cancer return to fully active lives by 1 year after diagnosis, although women who received adjuvant chemotherapy may be more likely to have some residual symptoms, such as sexual dysfunction (32,33). More research is clearly needed to characterize the recovery trajectory, in terms of both physical and psychological health, following a course of adjuvant chemotherapy.

Despite the high prevalence of breast cancer among older women, researchers have only recently focused on treatment questions in this patient group. Few randomized trials have included many women over 65 years of age (34–36). It is widely assumed that older patients are less tolerant of chemotherapy than younger patients. Although a few small studies have reported significantly increased toxicity in the elderly, recent larger studies provide evidence to the contrary. Crivellari et al. (37) studied the use of adjuvant CMF and tamoxifen in elderly women. Although women aged 65 years or older had greater hematologic and mucosal toxicity than younger women, quality-of-life measures suggested that the subjective burden of treatment was similar for older and younger patients. Begg and Carbone (38) examined 19 Eastern Cooperative Oncology Group studies that included a total of 780 patients aged 70 years or older. In comparison with younger individuals in the trials, older patients had increased hematologic toxicity; otherwise, the inci-

dence of severe toxic effects was similar between groups. In a more recent prospective study, Dees et al. (39) treated 44 women aged 35–79 years with early-stage breast cancer with four cycles of adjuvant AC chemotherapy. In this cohort, although myelosuppression was increased in older women, neutropenic complications, alteration in cardiac function, and change in quality-of-life scores were not significantly related to age. Pharmacokinetic analyses did not demonstrate age-related differences in the clearance of doxorubicin or cyclophosphamide. Although patients in these studies may represent a highly selected group, it is reassuring that the older patients appear to tolerate chemotherapy nearly as well as the younger patients. Additional research in this area is clearly warranted.

LONG-TERM OR SUSTAINED SIDE EFFECTS

In addition to the short-term side effects from chemotherapy, there are a number of sustained or long-term consequences of treatment. Some of these long-term effects, such as premature ovarian failure, are commonly seen in certain subgroups of patients. Others, such as secondary leukemia, are extremely rare consequences of treatment. Nevertheless, these rare effects must be considered in decision making about adjuvant therapy, particularly when the absolute benefits associated with treatment are of small magnitude.

Premature Ovarian Failure

Premature ovarian failure or premature menopause is a common consequence of adjuvant chemotherapy in premenopausal women. The risk of premature menopause appears to be related to patient age, the specific chemotherapeutic agents used, and the total dose administered. The effect of treatment duration and dose intensity, independent of total dose, is uncertain. While premature ovarian failure may have a beneficial effect on breast cancer prognosis (40), particularly in women with hormone receptor-positive tumors, early menopause may have important physiologic and psychosocial consequences. For women who wish to consider becoming pregnant after breast cancer, risk of infertility following chemotherapy is a major concern. Other problems related to premature ovarian failure include menopausal symptoms, such as hot flashes, genitourinary problems, and both psychological and psychosexual difficulties (33,41,42). Women who experience premature menopause have accelerated bone mineral density loss (43–46). Premature menopause may also contribute to increased cardiovascular morbidity, although data to support this concern in women with breast cancer are lacking. For many of these symptoms or complications, there are nonhormonal interventions available (47). However, patients commonly express concerns over menopausal symptoms and their bone and heart disease risk during longer follow-up.

Table 3 shows the proportion of women who experience premature menopause with adjuvant chemotherapy (48). The table is broken down by treatment regimen and age. The vast majority of women over the age of 40 years experience menopause after treatment with CMF or cyclophosphamide, epirubicin, and 5-fluorouracil (CEF). In women under the age of 40 years, the risk of ovarian failure from these regimens is lower but is by no means uncommon. MF has been reported to be associated with an approximately 10% incidence of premature menopause, but this has not been analyzed as a function of patient age. AC is associated with a lower incidence of premature menopause in

Table 3. Risk of premature menopause by regimen and age*

Regimen	Duration, mo	Incidence of amenorrhea, %	
		<40 y	≥40 y
CMF-based	6	31–38	81–96
	12	51–77	83–98
FEC	6	23	89
AC	3	13	57–63
MF	6	–10	

*Adapted with permission from Burstein and Winer in J. R. Harris' *Diseases of the Breast*, 2000 (48).

CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; FEC = 5-fluorouracil, epirubicin, and cyclophosphamide; AC = doxorubicin and cyclophosphamide; MF = methotrexate and 5-fluorouracil.

both younger and older women, probably because of the lower cumulative dose of cyclophosphamide with this regimen. The effect of adjuvant taxane therapy on premature ovarian failure is not well characterized. In one small retrospective study (49), the addition of paclitaxel to AC did not appear to substantially increase the overall risk of chemotherapy-related amenorrhea; however, larger studies are needed to make any definitive conclusions. In women under the age of 30 years, premature ovarian failure with any of the available regimens is distinctly uncommon. Three separate reports (50–52) have provided estimates for the incidence of premature ovarian failure in 20% or fewer women. In two of these studies (50,52), there were no patients under the age of 30 years who experienced premature menopause. In another report, Goodwin et al. (53) evaluated the incidence of ovarian failure in women who received no systemic therapy compared with those who received either chemotherapy or chemotherapy followed by tamoxifen. Young women (under the age of 30 years) had a very low incidence of menopause regardless of the therapy received. As expected, the incidence of chemotherapy-related amenorrhea increased with age.

Chemotherapy-related amenorrhea may be reversible in that some women will resume menstrual function months or years after treatment. However, the vast majority of women who remain amenorrheic 1 year after treatment will not regain ovarian function. The possibility of delayed (i.e., occurring even years after treatment) premature menopause has not been explored thoroughly. In the pediatric oncology population, there is evidence that adolescent girls who receive chemotherapy experience an earlier than expected menopause as they age (54). It is certainly plausible that a young woman who receives chemotherapy and does not experience chemotherapy-related amenorrhea will nevertheless go through menopause earlier than she would have in the absence of chemotherapy.

Weight Gain

Weight gain has been reported in 50% or more of women receiving adjuvant chemotherapy, with mean gains of 2.5–5.0 kg (55–58). More significant weight gain, as much as 10–20 kg, has been reported by some investigators in as many as 20% of patients. Weight gain appears to be more common in premenopausal women than postmenopausal women, and women who experience menopause with chemotherapy also seem to be at greater risk of weight gain (55–57). Regimens that are longer in duration may increase the risk of weight gain, and weight gain may be less common with the shorter AC regimen (59). Weight gain, particularly when substantial, can have a profound influ-

ence on a woman's physical health and psychological adaptation. In addition, retrospective studies (60–63) have suggested that weight gain may increase a woman's risk of disease recurrence.

The underlying cause of weight gain with chemotherapy is uncertain. For years it was assumed that weight gain occurred because women receiving chemotherapy simply ate too much. Studies that have monitored dietary intake have failed to support this view (57,64,65). Preliminary evidence suggests that weight gain may be caused by decreased physical activity during therapy (59,64,65). Studies (58,59,64–66) have also suggested that there may be changes in resting metabolic rate and that lean body mass can decline following a course of chemotherapy. Interventions focusing on exercise and on increasing lean body mass may help to ameliorate weight gain among women receiving adjuvant breast cancer chemotherapy (65).

Long-Term Cardiac Effects

Cardiotoxicity has been a major concern, since anthracycline-based regimens have been used more commonly in the adjuvant setting. The incidence of anthracycline-induced cardiac dysfunction increases with the increasing cumulative amount of anthracycline (either doxorubicin or epirubicin) administered. Other risk factors may include advancing age and a history of cardiac disease (67,68). In general, most adjuvant chemotherapy regimens restrict cumulative doses of doxorubicin to less than 360 mg/m² and of epirubicin to less than 720 mg/m²—doses thought to fall within a relatively safe range with clinically acceptable rates of cardiac complication. Valagussa et al. (69) reported a 0.8% incidence of congestive heart failure in a group of more than 500 women who received approximately 250 mg/m² of doxorubicin, with a median follow-up of 80 months. Zambetti et al. (70) performed a more detailed assessment of cardiac function in a group of 355 women who were disease free at a median follow-up of 11.5 years. Forty-four percent of the women received CMF only, and the remainder received CMF followed by doxorubicin, with a median cumulative doxorubicin dose of approximately 300 mg/m². Women were assessed by physical examination, history, electrocardiogram, and echocardiogram. Although clinical congestive heart failure was very rare in both groups, 8% of the patients receiving doxorubicin were characterized as having systolic dysfunction, defined as an ejection fraction of less than 55%. In contrast, fewer than 2% of the CMF group had evidence of systolic dysfunction. In a recent U.S. Intergroup trial using CAF in postmenopausal women, the reported incidence of congestive heart failure was approximately 2% (30). Of note, the patient population was somewhat older than in many adjuvant trials, and the total planned dose of doxorubicin was 360 mg/m².

The existing data concerning long-term cardiotoxicity are relatively reassuring, and the absence of clinical symptoms in the vast majority of patients is encouraging. However, the possibility of long-term subclinical systolic dysfunction, as seen in the Zambetti study, merits further investigation. Physicians can counsel women without pre-existing cardiac disease that the incidence of symptomatic cardiac problems with anthracycline-based regimens is extremely rare. There is reason to have some limited concern about the potential for very long term toxicity; it is not presently known whether anthracycline exposure increases the risk of cardiac compromise with subsequent cardiac stressors (e.g., hypertension) or a subsequent cardiac event (e.g.,

a myocardial infarction). In women with baseline cardiac dysfunction or in those who are at risk for compromise based on their medical history, it may be prudent to evaluate cardiac function before and after anthracycline-based adjuvant therapy, although data in support of this are limited.

Concern has been raised that breast/chest irradiation would increase the risk of cardiac toxicity. In a randomized trial of 5 versus 10 cycles of AC, there was an increased risk of cardiac events in the group of women who received 10 courses of treatment (median cumulative dose of doxorubicin, 442 mg/m²) (71). This effect seemed to be more pronounced in women who received high dose volume of cardiac irradiation. There appeared to be no excess cardiac risk in women who received five cycles of AC (median cumulative dose of doxorubicin, 225 mg/m²) with radiation therapy. In a retrospective analysis from Valagussa et al. (69), a total of four (0.8%) of 501 women treated with doxorubicin developed congestive heart failure, with a median follow-up in excess of 6 years; of the 114 women who received doxorubicin and left-sided breast irradiation, three (2.6%) developed congestive heart failure. Any increased concern with left-sided irradiation and the use of doxorubicin is probably less worrisome with the availability of modern radiation planning.

Chemotherapy-Associated Leukemia

Leukemia or myelodysplastic syndromes (MDSs) associated with adjuvant therapy are very rare, but devastating, complications of treatment. Curtis et al. (72) conducted a case-control study in almost 82 700 women who were treated for breast cancer during the 1970s and 1980s. On the basis of their work, the total dose of cyclophosphamide appears to be an important risk factor, with a substantially higher risk in women who receive more than 20 000 mg of the drug. With typical CMF regimens, which use significantly lower cumulative doses of cyclophosphamide, Curtis et al. (72) estimated that an additional five cases of leukemia would be seen in 10 000 women over the course of 10 years. Other investigators have used very different methodologies, making it difficult to compare across studies and with different regimens. There is some suggestion that the risk with anthracycline-based regimens may be greater than with classic CMF type regimens (12,15,73–78). With anthracycline-based regimens, the overall incidence of leukemia in women with breast cancer after standard-dose adjuvant therapy is approximately 0.1%–1.5% at 5–10 years' follow-up (12,15,30,77,79). In studies with 6 months of adjuvant anthracycline and cyclophosphamide therapy (e.g., CAF), the incidence of leukemia or MDS has been found to be as high as 1.5% (15,77), with an even greater risk associated with the addition of adjuvant radiation therapy (77). After four cycles of standard AC chemotherapy (cyclophosphamide at 600 mg/m² and doxorubicin at 60 mg/m² per cycle), the risk is probably quite low. This regimen was used as the standard arm of NSABP protocol B-22 (12), and the incidence of leukemia or MDS in this group was 0.1%, with a median follow-up of 5 years. Among women who received an increased dose or dose-intensive regimens of cyclophosphamide and doxorubicin on NSABP protocols B-22 and B-25 (12,77), the incidence of leukemia and MDS was higher. In both studies, there was no benefit in disease-free or overall survival observed among women who received the higher dose or dose-intensive regimens, and rates of leukemia and MDS ranged from 0.1% to 1.2%. It is reasonable to speculate that the higher doses of cy-

clophosphamide, up to 2400 mg/m² per cycle, may have contributed to the higher frequency of leukemia and MDS in these studies. In the preliminary report of the NSABP B-28 trial (23), five (approximately 0.3%) cases of leukemia developed in the approximately 1500 patients who received standard-dose AC followed by paclitaxel. Whether there is any additional increase in risk with the addition of the taxanes is unknown.

The latency period and cytogenetic abnormalities appear to be different with doxorubicin-induced leukemia than those that arise after exposure to cyclophosphamide alone (75). Leukemias that are associated with exposure to alkylating agents typically present 5–7 years after treatment and are frequently preceded by an MDS. Topoisomerase inhibitors, such as anthracyclines, can give rise to secondary leukemias 6 months to 5 years after therapy. There are no methods of screening for these disorders in survivors of breast cancer, although they should be considered in the evaluation of patients in whom cytopenia develops after the treatment of breast cancer. Because of the rarity of leukemia after adjuvant therapy, concern about this complication seems most reasonable in women who are at low risk of breast cancer recurrence and who are likely to derive a very small benefit from adjuvant chemotherapy.

Cognitive Dysfunction

Cognitive dysfunction after adjuvant therapy has received increasing attention in both the medical and lay literature in recent years. Three studies have been published (80–82) in which women who had received or were receiving chemotherapy underwent neuropsychiatric testing and were compared with a control group. Schagen et al. (81) evaluated 39 women who were approximately 2 years out from six cycles of CMF (with or without subsequent tamoxifen) and compared them with 34 women who had received local therapy only. Twenty-eight percent of the CMF group, compared with 12% of the control subjects, had evidence of cognitive dysfunction, predominantly characterized by difficulties with concentration, memory, word-finding, and motor-testing. Furthermore, hormonal therapy did not appear to influence patients' self-reports of symptoms or cognitive function. In a study by van Dam et al. (80,83), a dose-effect relationship was seen between chemotherapy and cognitive dysfunction. At a mean of 2 years since the completion of last nonhormonal therapy, impaired cognitive dysfunction was seen in 32% of the patients treated with high-dose chemotherapy, in 17% of the patients treated with standard-dose chemotherapy, and in 9% of the women with stage I breast cancer who did not receive chemotherapy. Brezden et al. (82) surveyed a group of 31 women receiving chemotherapy, another group of 40 women who had received chemotherapy in the past, and a group of healthy control subjects. Impaired cognition was seen more frequently in women on active treatment compared with control subjects, and cognitive difficulties did not appear to be related to anxiety or depression. While these results are provocative, it is important to note that, in two of the studies (80,81), there was no association between self-reports of cognitive dysfunction and scores on the formal testing; the women who complained of cognitive difficulties were not the same women who performed poorly on the testing. Furthermore, in none of these studies were patients assessed longitudinally to assess for change in functioning with therapy. Anecdotally, many patients complain of what has commonly been termed "chemo brain," with complaints of forgetfulness and difficulty concentrating.

The possibility of persistent impaired cognition is of great concern to patients as they make decisions about adjuvant treatment, but neither the anecdotes nor the research studies conducted to date permit any firm conclusions. Prospective longitudinal studies are warranted to pursue the hypothesis that cognition may be impaired in women following adjuvant chemotherapy.

SUMMARY AND CONCLUSIONS

How should women and their physicians use information about side effects to make decisions about adjuvant therapy? A woman with a new diagnosis of breast cancer needs to consider her risk of disease recurrence and death in the absence of therapy, the potential benefit of chemotherapy, and her post-treatment risk of recurrence and death. For an individual woman, it is the absolute, not the relative benefit, of therapy that is important. This benefit needs to be considered in the context of the short-term and long-term side effects from treatment.

In making these decisions, women and their physicians need to know the frequency, duration, and severity of side effects. This information, at least for broad groups of women, is available. Unfortunately, for many of the side effects, clinicians have relatively little ability to predict who is at greater or lesser risk of experiencing a given adverse effect. Improving the ability to predict an individual woman's risk of both long- and short-term side effects with various treatments will allow her to make an even more informed decision regarding therapy. Perhaps even more importantly, the impact of side effects on a woman's ability to carry on her daily activities has not been well evaluated. Many women want to know whether they will be able to continue to care for their families, work, and pursue the activities they enjoy—that is, continue with their lives, despite treatment. Future research focusing on this aspect of patient care is needed.

Decisions about adjuvant chemotherapy are complex. No woman with localized breast cancer can know that she definitely will experience a recurrence in the absence of therapy, and even if she did, there is no guarantee that treatment will prevent such a recurrence. For that matter, even women with very early stage disease are at some risk of a systemic recurrence after local therapy alone. The potential benefits of adjuvant treatment need to be considered in conjunction with the risk of short-term and long-term side effects. Not only should the patient and physician consider the frequency and intensity of the side effects, but they must also consider how any particular side effect may impact an individual woman's life. Decisions about adjuvant treatment are often not clear-cut, but by weighing the advantages and disadvantages of a course of treatment, patients and their physicians can hope to make informed and thoughtful choices.

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Decision-Making Process—Communicating Risk/Benefits: Is There an Ideal Technique?

Mark Levine, Tim Whelan

During the last decade, there have been major advances in the treatment of early-stage breast cancer. The decisions a patient now must make concerning her treatment are often difficult and complex, e.g., mastectomy versus lumpectomy plus breast radiation therapy, adjuvant chemotherapy and/or hormonal therapy versus no further treatment, regional radiation therapy or no regional radiation therapy.

In the past, physicians tended to make decisions for patients with little patient input. More recently, women have indicated the need for more information about their disease and a desire to be involved in decisions about their care (1). Degnar et al. (2) examined the preferences of 1012 women with breast cancer for participation in treatment decision making. Twenty-two percent of the women wanted to select their own cancer treatment (active role), 44% wanted to select their treatment collaboratively with their physician (collaborative role), and 34% wanted to delegate this responsibility to their physician (passive role). Education and age influenced the preferred role in decision making.

In general, the patient/physician encounter will involve several stages, including exchange of information between the doctor and the patient, deliberation, and decision making (3). At one extreme is a paternalistic model, where information flows in one direction—from the doctor to the patient—and the doctor alone makes the decision. At the other extreme is the informed model, where again, information flows mainly in one direction, but the patient alone makes the decision. In between these models is the shared model, in which the doctor and patient share all stages of the decision-making process simultaneously. There is a two-way exchange of information, both doctor and patient reveal treatment preferences, and both agree on the decision to implement. It is the shared model for decision making that provides the foundation for the use of decision aids. Studies have demonstrated that the majority of women with breast cancer and their physicians prefer shared models for decision making (4).

Studies have suggested problems with the traditional physician/patient encounter, particularly with the transfer of information and patient involvement in decision making (5,6). Siminoff et al. (5) studied 100 consecutive physician–patient encounters for adjuvant chemotherapy in women with early breast cancer to assess the consultative approach. They observed that the communication pattern, particularly that of the physician, was independent of characteristics of the patient and the severity of her disease. The risks and benefits of treatment were discussed, but the physician exchanged little in the way of specific information, and the impact of treatment on the patient's lifestyle and emotional state often was not routinely addressed. Not surprisingly, the majority of patients (60%) overestimated their chance of being cured by 20% or more and underestimated the likelihood of severe common side effects by a similar percentage. Although patients were given alternative options, physicians generally recommended one treatment, and this had a definite influence on the patient's decision. Rimer et al. (6) reviewed 116 consultations regarding adjuvant chemotherapy between physicians and

patients. Clinicians, on average, told patients less than 70% of the information relevant to their disease and treatment.

On the basis of these considerations, researchers and clinicians have responded by investigating better ways of transferring information to patients and supporting them in decision making. Decision aids have been defined as “interventions designed to help people make specific and deliberative choices among options by providing information on the options and outcomes relevant to the person's health status” (1). Examples of decision aids are written materials, computer-based programs, video programs, audio-guided workbooks, and decision boards. These methods differ from traditional patient education materials both in that they provide an explicit presentation of different treatment options with the associated benefits and risks and in that the information provided is often tailored to the individual characteristics of the patient and her disease.

O'Connor et al. have conducted a systematic review of decision aids in various cancers (1) and other health conditions (7). The results of studies evaluating these decision aids demonstrated that they are acceptable to patients and can improve knowledge and make patients more comfortable but did not appear to have a consistent impact on patient satisfaction. There have been relatively few studies of decision aids in breast cancer. We will review the use of decision aids in women with early breast cancer who are faced with treatment options. We will not consider decision aids for early detection of breast cancer or for communicating risk for prevention.

SURGERY: MASTECTOMY VERSUS BREAST CONSERVATION THERAPY

There have been five studies examining the use of decision aids in the surgical management of breast cancer (Table 1). These have all been of relatively small sample size. In the first study, Chapman et al. (8) randomly assigned 82 undergraduate psychology or nursing students to view either a videodisc or a brochure. Subjects were asked to consider a hypothetical choice of lumpectomy versus mastectomy for early breast cancer. The videodisc provided information as well as an interview with patients. Although the videodisc and brochure both increased patient knowledge, there was no difference between the interventions. Viewing the videodisc resulted in a shift in preference to lumpectomy.

In a study by Street et al. (9), women who had recently had a positive biopsy for breast cancer were randomly assigned to view either a multimedia program (consisting of an interactive

Affiliation of authors: Cancer Care Ontario Hamilton Regional Cancer Centre, Department of Medicine, McMaster University, Hamilton, ON, Canada

Correspondence to: Mark Levine, M.D., Clinical Research Institute, Faculty of Health Sciences, Rm. 2E5, McMaster University, 1200 Main St. W., Hamilton, ON L8N 3Z5, Canada (e-mail: mlevine@mcmaster.ca).

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Table 1. Mastectomy versus lumpectomy

Investigators (reference No.)	Intervention	Design*	Results		
			Knowledge	Choice	Other
Chapman et al. (8)	Videodisc versus brochure	RCT	Improved with both†	Lumpectomy	—
Street et al. (9)	Multimedia versus brochure	RCT	Improved with both†	Lumpectomy (NS)‡	No difference in optimism
Goel et al. (10)	Audiotape + workbook versus brochure	RCT	No difference	—	Lower decreased conflict in aids group§
Whelan et al. (11)	Decision board	Before-after	—	Shift to mastectomy	—
Molenaar et al. (12)	CD-ROM	Before-after	—	Shift to lumpectomy	Lower decision uncertainty

*RCT = randomized control trial.

†Difference between groups not statistically significant.

‡NS = not statistically significant.

§Healthy volunteer.

computer) or a brochure before the consultation with a surgeon. Knowledge and optimism questionnaires were administered at baseline assignment, after the assignment, and after the physician consultation. There were 30 patients in each group. Seventy-six percent of the computer group chose lumpectomy versus 58% who chose lumpectomy in the brochure group; this difference was not statistically significant. Both the brochure and multimedia interventions increased patient knowledge, and there was a trend in favor of the multimedia program ($P = .07$). No difference in optimism was detected between groups.

Goel et al. (10) randomly assigned 38 surgeons in clusters to the use of an audiotape and workbook or an information brochure alone. One hundred sixty-four patients were enrolled in this study. There was no difference detected between the decision-aid and brochure groups in terms of knowledge and anxiety. There was a trend for lower decisional conflict in the decision-aid group, but the difference was not statistically significant.

Whelan et al. (11) developed a decision board for use by community surgeons and their patients regarding the choice of mastectomy versus lumpectomy. The decision board consists of a visual aid and written material. A clinician administers this instrument during the patient consultation. Information is presented in an interactive step-by-step fashion. The instrument was administered to 175 women with breast cancer at the decision-making point. Ninety-eight percent of the patients reported that the board was easy to understand, and 81% indicated that it helped them to make a decision. In 90% of the consultations, surgeons found the board easy to use and helpful. In a before-after design, the rate of mastectomy increased from 12% to 27% ($P < .01$). The surgical decision board is currently being evaluated in a randomized trial using a cluster randomization design. Surgeons are randomly assigned the use either of the decision aid plus usual consultation or of the usual consultation.

Finally, Molenaar et al. (12) developed an interactive computer program (CD-ROM) as a decision aid. This was acceptable to 96 women with early breast cancer. Using a before-after design, there was a shift in treatment preference for breast conservation therapy. In addition, posttest levels of decision uncertainty were significantly lower after using the interactive program ($P < .01$).

BREAST IRRADIATION FOLLOWING LUMPECTOMY

Whelan et al. (13) developed a decision board for use in women with lumpectomy who were undergoing breast irradiation. This study was initiated a number of years ago following completion of a randomized trial comparing the results of radiation

therapy versus no radiation therapy in the lymph node-negative women who had undergone lumpectomy. The concept at that time was to try to identify a group at low risk for recurrence who might be spared radiation therapy. Consecutive cohorts of lymph node-negative women who had undergone lumpectomy were studied. Patients at high risk for systemic recurrence were excluded from the study. Twenty-three women underwent a consultation by the radiation oncologist alone. The next 29 women underwent consultation and, in addition, the radiation oncologist used a checklist that served as a reminder to cover a number of important information points concerning breast irradiation in the interview. Following this, 30 patients underwent the usual consultation and, in addition, were administered a decision board. The use of the decision board increased knowledge compared with the consultation alone and with the consultation plus checklist. The instrument also appeared to facilitate shared decision making and empower women in the decision-making process. Ninety-seven percent of patients in the board group felt that they were offered a choice concerning breast irradiation compared with 70% in the consultation group ($P = .02$). Eighty percent of patients in the board group reported making a decision without a formal recommendation from their physician, compared with only 8% in the consultation group ($P < .01$).

ADJUVANT CHEMOTHERAPY

Levine et al. (14) developed a decision board for use in women with high-risk lymph node-negative breast cancer who were considering adjuvant chemotherapy. The validity and reliability of the board were established in healthy volunteers. The instrument was found to be acceptable and helpful in 37 newly presenting women with high-risk lymph node-negative breast cancer who were considering adjuvant chemotherapy. This type of board has been evaluated in a randomized trial. One hundred seventy-six women with lymph node-negative breast cancer were randomly assigned to either the medical oncology consultation or the medical oncology consultation plus the decision board. The outcome measures include knowledge, satisfaction, and treatment choice.

Ravdin et al. (15) have developed a decision aid in the form of a simple-to-use computer program. This program is designed to produce prognostic estimates of outcome both with and without therapy based on estimates of individual patient prognosis and estimates of the efficacy of different adjuvant therapy options. The computer program can present this information on the screen for the physician and as printed pages for use with pa-

tients. This decision aid has been evaluated in a clinical trial in which 44 doctors were randomly assigned to the usual consultation approach versus the consultation plus decision aid. Four hundred four patients participated in this trial. The endpoints include knowledge, satisfaction, and treatment choice.

CONCLUSION

In general, decision aids have been shown to improve patient knowledge and make patients more comfortable with treatment decision making. There have been relatively few studies of decision aids in patients with early-stage breast cancer. Nonetheless, it appears that women with early breast cancer find decision aids helpful in decision making and that decision aids can improve these women's knowledge concerning treatment options. Decision aids facilitate shared decision making in women with breast cancer. Physicians and surgeons can use decision aids in their practice and may find them helpful. Future research is required to determine whether decision aids can improve such outcomes as patient satisfaction, quality of life in the long term, and unexplained practice variation. In addition, research will determine whether particular decision aids are better than others or whether a particular aid is better for a particular patient group or intervention.

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Patient Preferences for Adjuvant Chemotherapy of Early Breast Cancer: How Much Benefit Is Needed?

R. John Simes, Alan S. Coates

Adjuvant chemotherapy for early-stage breast cancer has been shown to delay recurrence and improve survival. However, the benefits are modest and must be balanced against the adverse treatment effects. We assessed the size of the survival benefit needed to justify the toxicity of chemotherapy, based on the preferences of women who had previously received adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF). We also attempted to identify circumstances in which larger survival gains would be needed. In semistructured interviews, 104 women who had received adjuvant CMF chemotherapy were asked to rate the survival benefit that would justify 6 months of such treatment, using a series of hypothetical trade-offs between shorter survival without treatment and longer survival with treatment. Similar preferences were sought for a greater probability of 5-year survival. Most patients considered 6 months of adjuvant CMF chemotherapy worthwhile for relatively modest survival gains: 77% considered an increase of from 5 to 6 years worthwhile, 74% thought an increase of from 15 to 17 years worthwhile, and more than 70% considered such treatment justified for a 5% greater chance of living 5 or more years. Smaller survival benefits were needed for women who had experienced less toxicity ($P = .01$), had not received initial radiotherapy ($P = .01$), had better social support ($P = .02$), and had others at home dependent on their support ($P = .0001$). Modest survival benefits are sufficient to justify adjuvant cytotoxic chemotherapy for most women with early-stage breast cancer. Individual preferences are important when weighing trade-offs between survival and adverse treatment effects. [J Natl Cancer Inst Monogr 2001; 30:146-52]

Chemotherapy used as an adjuvant treatment for patients with operable breast cancer has been shown clearly to reduce the chance of the breast cancer recurrence and to improve overall survival (1). While the evidence of benefit is clear, the magnitude of survival benefit has been modest. Greater absolute benefits occur among younger patients and among those at greater risk of relapse, such as women younger than 50 years with positive axillary lymph nodes, for whom an increase in the chance of 10-year survival of approximately 11% might be expected. By comparison, for older women without lymph node involvement, the survival benefit is less clear and may be as little as 2%-3% (1). These modest improvements in survival rate might translate into gains in life expectancy of 1-3 years (depending on assumptions made about long-term effects).

The benefit of improved survival and reduced risk of recurrence must be balanced against the side effects of chemotherapy, such as hair loss, nausea, tiredness, and risk of infection, with a resultant detriment to quality of life (2,3). Furthermore, these adverse effects usually occur early and are obvious to the patient, whereas the benefits of treatment may be delayed and less

evident for the person. In this setting, preferences of individual patients on the relative importance of these outcomes may be crucial to optimal decision making about whether to give or withhold treatment. The views of patients who have actually experienced such treatment may be particularly helpful in deciding whether or not to treat future patients who are similar.

Eliciting patient preferences to guide decision making is an important but complex process. Answers may vary according to how questions are asked and under what circumstances, as well as by whose views are sought (4,5). Surrogate decision makers may give answers that are systematically more conservative than those of patients (5-8). Healthy volunteers know neither the anxieties of having cancer nor the actual side effects of treatment. Even women with breast cancer who have not experienced chemotherapy may make judgments based on the worst possible side effects rather than on a reasonable average expectation (9).

We therefore designed a study to ask women who had experienced adjuvant chemotherapy for early breast cancer what survival benefit would justify the treatment as they had experienced it (10). The primary aim was to assess the size of the survival benefit needed to justify the toxicity of adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy. The study was also designed to identify possible patient and disease factors affecting whether larger survival gains were needed to justify adjuvant chemotherapy.

PATIENTS AND METHODS

Patients

Women who had received at least three cycles of CMF chemotherapy as adjuvant treatment after local treatment for operable breast cancer and who were attending a clinic at the Royal Prince Alfred Hospital, Sydney, Australia, from November 1986 to December 1987 were approached about participating in the study. Patients who, having started such therapy, withdrew from it either by their own choice or by the decision of their doctor were also eligible to participate. Consent was obtained both from the patient and her doctor. Of 129 patients considered for participation in the study, nine were excluded because of insufficient comprehension of English, five were considered too ill to participate, three were geographically inaccessible, two died before the interview, and two were not asked. Thus, 108 patients

Affiliations of authors: R. J. Simes, National Health and Medical Research Council Clinical Trials Centre, University of Sydney, Sydney, New South Wales, Australia; A. S. Coates, Department of Public Health and Community Medicine, University of Sydney, and Australian Cancer Society, Sydney, New South Wales, Australia.

Correspondence to: John Simes, M.D., F.R.A.C.P., National Health and Medical Research Council Clinical Trials Centre, Mallett Street Campus, University of Sydney, New South Wales 2006, Australia (e-mail: enquiry@ctc.usyd.edu.au).

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were asked to participate; 104 patients consented and completed the initial interview.

Patient Interviews

All 104 participating patients underwent a semistructured interview with one of two trained observers not involved in the patient's care at least 3 months after the completion of the adjuvant chemotherapy. Information was also obtained at this time on each patient's sociodemographic, disease, and treatment characteristics as well as on toxic effects experienced from chemotherapy. Patient preferences for adjuvant chemotherapy were elicited by using a series of hypothetical trade-offs between a lesser survival period without treatment and a greater survival period with adjuvant treatment plus its associated toxicity.

Time Trade-off Questions

Patients were presented with hypothetical scenarios of the general form: "Suppose that without treatment you would live 5 years. Based on your own experience of chemotherapy, what period of survival would make 6 months of initial treatment worthwhile?" Patients were then asked to express a preference between 5 years' survival without treatment or a longer period of survival beginning with 6 months of adjuvant treatment, using a series of cards to represent each scenario. The second card with the longer period was then altered until the patient considered it to be roughly of equal value to the 5 years without treatment. This was referred to as the 5-year trade-off. A similar sequence was then followed to establish equivalence for a hypothetical patient at lower risk with an expectation of 15 years' survival without treatment. This was referred to as the 15-year trade-off.

Survival Rate Questions

These questions were similar to the time trade-off approach but expressed the outcome of treatment in terms of percentage chance of remaining alive at 5 years. Patients were asked to express a preference between a 65% chance of 5-year survival without adjuvant chemotherapy and a higher chance of 5-year survival with 6 months of adjuvant chemotherapy. The higher chance of 5-year survival was again varied until it was considered roughly equal to the 65% chance of 5-year survival without treatment. Similarly, patients were asked to express a preference between 85% chance of 5-year survival without adjuvant chemotherapy and a higher chance with 6 months of adjuvant chemotherapy.

Retest Interviews

Where possible, patients were interviewed 3–6 months after the initial interview. Thirty-nine patients were not interviewed a second time: 12 patients refused a second interview, four had died in the interval, six were excluded because of poor comprehension or anxiety at the first interview, and 17 were lost to follow-up before the planned second interview. Results of the repeat interviews were used to assess the reliability of the measures used and to assess changes in preference over time.

Interview Methods

To assess possible framing effects of questions, the sequence in which alternatives were offered was randomly assigned. For the 5-year trade off questions, patients were initially offered a

period of 6 or 10 years with treatment versus 5 years without treatment. In either case, the options were altered in response to the patient's reply until equivalence was established. For survival rate questions, framing effects were also assessed by randomly offering initial options at the high or low end of the expected response range, beginning with an extra 1% or with an extra 10%.

Statistical Methods

The results of the time trade-off and survival rate questions were skewed, and no effective normalizing transformation was available, so primary analyses were nonparametric. The cumulative proportion of those accepting chemotherapy as worthwhile was plotted for each size of survival benefit. The comparison of preferences for major groups was undertaken by using the Kruskal–Wallis test. Patient and disease factors predicting individual preferences were assessed in multivariate linear regression analysis, in which the outcome used was a normal score associated with the rank of each individual's time trade-off responses (11). To avoid the problems of using multiple outcomes, we specified *a priori* the total score of the two time trade-off questions as the primary outcome in these analyses: the time trade-off total. All variables in Table 1, except those with low frequency, were included in the multivariate analyses.

RESULTS

Patient and Treatment Details

The patient and disease characteristics of the 104 women interviewed are shown in Table 1. The median age was 49 years (range, 25–67 years). Almost all of the women were treated with mastectomy and at least six cycles of CMF chemotherapy. CMF chemotherapy was associated with some severe nonhematologic toxicity in 15% of the patients and with moderate or severe nonhematologic toxicity in 62% of the patients (Table 2).

Patient Interviews

Fifty-five interviews were completed by the first interviewer; the remaining 49 were completed by the second interviewer.

Reliability

Test–retest reliability was assessed by Spearman's rank correlation between first and second interview in 65 patients. The correlation coefficient was .68 for the 5-year time trade-off question and .64 for the 15-year time trade-off. For the 65% and 85% survival rate questions in interviews with 63 patients, the correlation coefficients were .63 and .69, respectively. These figures somewhat overestimate reliability, since some patients were excluded from a second interview because they had had problems at the first interview. There was no systematic change in the answers to time trade-off questions, but there was a statistically significant change to a larger increment in survival rate (by an extra 1%–2%) needed for adjuvant treatment in second interviews ($P \leq .003$). Among patient responses to the 5-year trade-off, the correlation was higher when the retest interview was done by the same interviewer (0.75) than when it was done by the other interviewer (0.63).

Framing and Other Effects

No statistically significant difference was observed in any of the end points selected as a result of the sequence in which

Table 1. A. Baseline patient characteristics

Patient characteristic	Patients (n = 104), %
Age at interview, y	
<50	57
50-59	30
≥60	14
Married	73
Educational level	
Primary	12
Secondary	63
Tertiary	25
Employment	
Full	31
Partial	21
None	48
No. of others at home	
None	9
1	35
2	22
≥3	35
Support needed by dependents	
Nil	43
Partial	37
Full	20
Support available to patient	
Nil	7
Partial	31
Full	31

Table 1. B. Disease and treatment details

Disease characteristic	Patients (n = 104), %
Tumor stage at diagnosis	
T1	16
T2	74
T3	8
Positive axillary lymph nodes	
0	3
1-3	50
≥4	44
Disease status at interview	
Disease free	81
Local relapse	5
Distant relapse	11
Both	4
Time from diagnosis to interview, y	
≤1	14
≤3.5	50
>3.5	50
Time from chemotherapy end to interview, y	
≤1	27
≤3.0	50
>3.0	50
Surgery	
Lumpectomy	9
Mastectomy	91
Radiation therapy	18
Adjuvant endocrine therapy	18
Oral CMF*	96
Intravenous CMF*	4
No. of cycles given	
<6	10
6	61
7-14	30
Percentage dose received of total planned	
≤75	41
>75	59

*CMF = cyclophosphamide, methotrexate, and 5-fluorouracil combined.

Table 2. Worst toxicity grade reached with adjuvant chemotherapy*

Toxicity type	None, %	Mild, %	Moderate, %	Severe, %
Alopecia	19	46	29	6
Mucositis	55	23	21	1
Nausea and vomiting	12	37	43	9
Hematologic	30	33	34	4
Other	55	37	7	2
Worst nonhematologic	3	20	62	15

*Based on World Health Organization criteria.

alternatives were offered. Furthermore, in the multivariate analysis, preferences did not differ statistically significantly according to which interviewer was involved, how long after diagnosis or treatment the interview was undertaken, or whether the patient's breast cancer had recurred before the interview.

Patient Preferences

A large majority of the patients felt that relatively modest improvements in survival duration or in the percentage chance of 5-year survival would justify 6 months of the treatment they received. This was true both in the relatively optimistic scenarios, with an untreated survival duration of 15 years or 5-year survival rate of 85%, and in the less favorable scenarios, with an untreated survival expectation of 5 years or a 65% 5-year survival rate.

Details of the percentage of responding patients accepting that treatment would be worthwhile at various trade-off points are displayed in Fig. 1 and in Tables 3 and 4. A majority of the patients considered relatively small survival gains to be sufficient to justify treatment, and a substantial minority of the patients considered only 6 months of extra survival enough. Thus, 46% of patients considered a survival period of 5.5 years with treatment equivalent to 5 years without such treatment, and 39% of patients would accept a similar increment even with an expected survival of 15 years (Fig. 1, A; Table 3). Furthermore, 77% considered an increase from 5 to 6 years worthwhile, while 74% thought an increase from 15 to 17 years worthwhile. Larger survival gains were needed for the scenario with the longer (15-year) survival, indicating that women were discounting benefits of treatment that were appreciably delayed. Results of the survival percentage trade-off were more extreme, with almost one-half of the women judging a 1% improvement in 5-year survival probability as justifying treatment, whether the expected 5-year survival without treatment was 65% or 85%. The minimum extra survival at which a majority of patients would accept adjuvant treatment was 1 additional year, whether the baseline was set at 5 years or at 15 years (Table 3), with an additional 2% for each of the survival rate trade-offs (Table 4). Importantly, for some women even very large survival benefits would be insufficient to justify the toxicity of treatment.

Factors Affecting Patient Preferences

In a multivariate analysis of all prespecified baseline factors, statistically significant predictors of stronger preferences for adjuvant treatment (with smaller survival benefits needed) were less toxicity from chemotherapy ($P = .01$), not receiving radiotherapy as part of the initial treatment ($P = .01$), full-dose chemotherapy ($P = .02$), having better social support ($P = .02$), and having others at home dependent on their support ($P = .0001$).

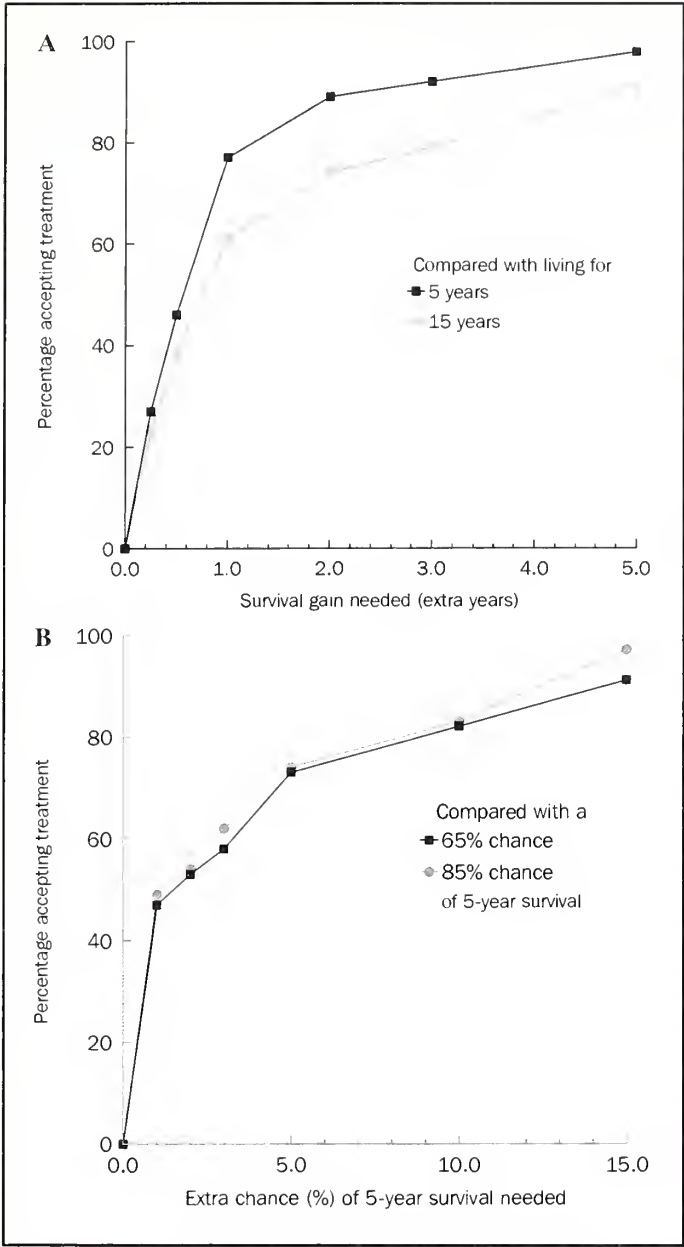


Fig. 1. A) Proportion of patients who would consider the extra years in survival plotted sufficient to accept adjuvant chemotherapy compared with 1) 5 years or 2) 15 years of survival without such treatment. **B)** Proportion of patients who would consider the extra chance of 5-year survival plotted to be sufficient to accept adjuvant chemotherapy compared with 1) a 65% chance or 2) an 85% chance of living at least 5 years.

Support Required by Dependents of the Patient

Support required was categorized as none (46 patients), partial (37 patients), and full (20 patients). This was strongly associated with the time trade-off endpoints selected by the women. Patients whose dependents required full or partial support were more likely to judge treatment acceptable and needed smaller increments in survival to justify treatment (Fig. 2; $P = .002$ for 5-year trade-off; $P = .0004$ for 15-year trade-off, and $P = .0004$ for time trade-off total). No such effect was seen in the survival rate questions, perhaps because most patients selected similar small-percentage increments. This factor remained statistically significant in the primary main multivariate analysis, based on trade-off total ($P = .0001$).

Table 3. Time trade-off decision points: minimum expected additional survival needed for patients to accept adjuvant chemotherapy treatment

Expected additional survival needed for patient to accept adjuvant chemotherapy	Preference, compared with living without having adjuvant therapy for			
	5 y*		15 y†	
	No.	Cumulative %	No.	Cumulative %
0 ≤ 3 mo	28	27	23	23
3 ≤ 6 mo‡	44	46	17	39
6 ≤ 12 mo	32	77	22	61
1 ≤ 2 y	13	89	13	74
2 ≤ 3 y	3	92	6	79
3 ≤ 5 y	6	98	12	91
5 ≤ 15 y	1	99	0	91
No increase enough	1	100	9	100

*Preference of one patient not obtained.
 †Preferences of two patients not obtained.
 ‡3 ≤ 6 months indicates that more than 3 months but not more than 6 months additional survival is needed to justify adjuvant chemotherapy.

Table 4. Survival percentage trade-off decision points: minimum additional chance of 5-year survival (%) needed for patients to accept adjuvant chemotherapy treatment

Additional chance, %, of 5-y survival needed for patient to accept adjuvant chemotherapy	Compared with a chance of living at least 5 y without having adjuvant chemotherapy			
	65% Chance*		85% Chance†	
	No.	Cumulative %	No.	Cumulative %
≤ 1	47	47	48	49
1 ≤ 2‡	5	53	5	54
2 ≤ 3	5	58	8	62
3 ≤ 5	15	73	12	74
5 ≤ 10	9	82	8	83
10 ≤ 15	9	92	14	97
15 ≤ 25	7	98	0	97
No increase enough	2	100	3	100

*Preferences of five patients not obtained.
 †Preferences of six patients not obtained.
 ‡1 ≤ 2 months indicates that more than an additional 1% chance but not more than an additional 2% chance of 5-year survival is needed to justify adjuvant chemotherapy.

Support Available to the Patient

Patients to whom full support was available from others accepted smaller increments in survival as justifying treatment than did those patients with partial or no support. This was statistically significant for the 15-year trade-off ($P = .01$) and for trade-off total ($P = .04$). It remained statistically significant in the multivariate analysis ($P = .02$).

Treatment-Related Toxicity

Univariate analysis showed statistically significant associations between 5-year trade-off and mucositis ($P = .04$), while for 15-year trade-off hematologic toxicity and mucositis were statistically significant (both $P = .04$). In the multivariate analysis based on trade-off total, the summary factor describing any nonhematological toxicity remained independently statistically significant ($P = .01$). As expected, patients experiencing worse toxicity demanded greater improvements in survival to justify treatment.

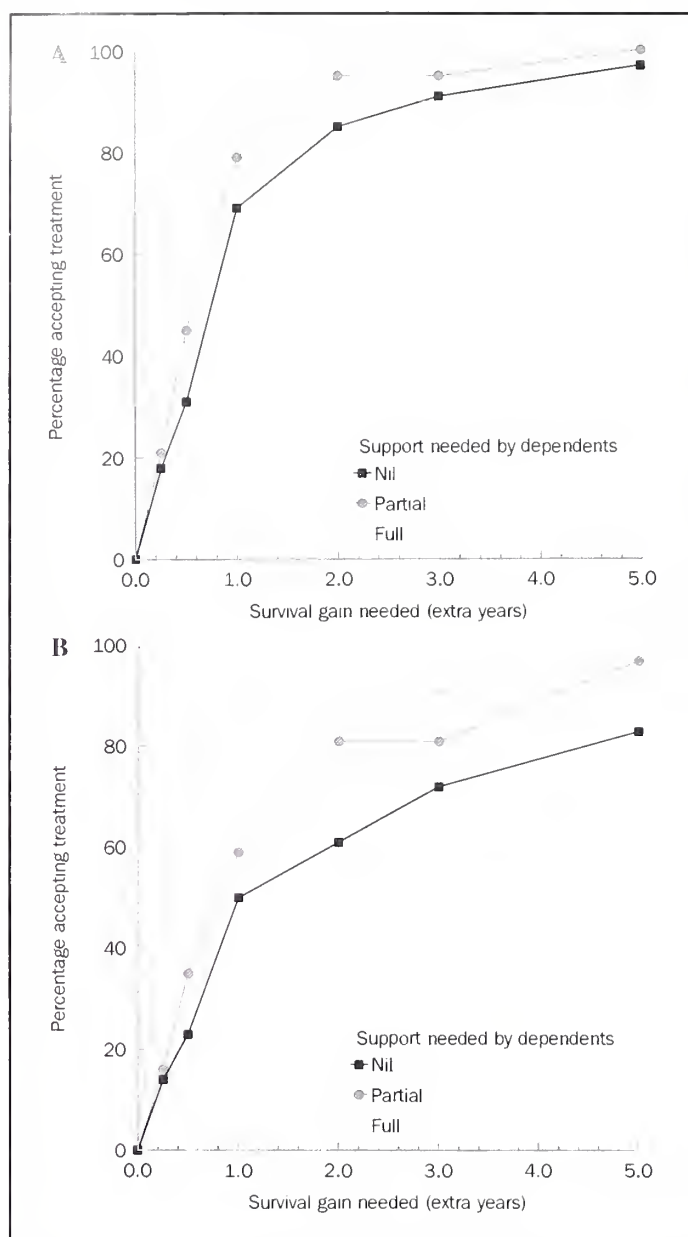


Fig. 2. Variation in patient preferences for adjuvant chemotherapy according to the amount of support required from the patient for dependents at home: 1) no support needed, 2) partial support needed, or 3) full support needed. Proportion of patients willing to accept adjuvant chemotherapy for a given increase in survival duration compared with 5 years (panel A) or 15 years of survival (panel B) without such treatment.

Dosage Reduction During Chemotherapy

Patients whose chemotherapy dosage was reduced to 75% or less of the total planned dose demanded longer survival increments than did those receiving a higher dosage ($P = .05$ for 5-year trade-off, 15-year trade-off, and trade-off total). This factor was independent of recorded toxicity in a multivariate analysis ($P = .02$).

Initial Radiotherapy

Patients whose initial adjuvant treatment included radiotherapy as well as chemotherapy required larger increments of survival to justify treatment. This was independent of other

factors in the multivariate analysis based on trade-off total ($P = .01$).

Factors Not Predictive of Patient Preferences

No association was observed between the trade-off total and patient age, educational level, or employment status; the time between treatment and interview; the use of concurrent adjuvant endocrine therapy; the occurrence of relapse; or the use of any particular modality (including further chemotherapy) for the treatment of relapse.

DISCUSSION

Adjuvant chemotherapy is now widely used to treat women with early breast cancer with the aim of preventing recurrence and improving overall survival. However, the benefits of adjuvant chemotherapy may be difficult to quantify and weigh against the adverse effects of treatment. For younger women at high risk of recurrence, such as those with axillary lymph node involvement, the gains in survival are larger, with an improvement of about 11% in the 10-year survival rate (see Table 5). For those at lower risk, the gains in survival are smaller. In women younger than 50 years, the improvement in 10-year survival is estimated at 7%, and for women older than 50 years the gain is in the range of 2%–3% (1).

When considering benefits in terms of prolongation of time to relapse and overall survival, Cole et al. (12) estimate that for younger women (<50 years old), polychemotherapy adds an additional 5.4 months of survival and an additional 10.3 months of relapse-free survival within the first 10 years of follow-up. For older women (50–69 years old), the gains are smaller, with an estimated additional 2.9 months of overall survival and 6.8 months of disease-free survival. This would translate into an additional 6–8 months of quality-adjusted survival for younger women and an extra 3–5 months for older patients, accumulated within 10 years (Table 5). These estimates ignore longer term

Table 5. Estimated benefits of adjuvant polychemotherapy for early breast cancer

Outcome	Subgroup, y	Benefit
Increase in 10-y survival rate*		
Women aged <50		
Axillary lymph node positive		11.5%
Axillary lymph node negative		7.1%
Women aged 50–69		
Axillary lymph node positive		3.2%
Axillary lymph node negative		2.4%
Survival gain within 10 y	<50	5.4 mo
	50–69	2.9 mo
Disease-free survival gain within 10 y†	<50	10.3 mo
	50–69	6.8 mo
Extra quality-adjusted survival within 10 y‡	<50	6–8 mo
	50–69	3–5 mo
Potential survival gain after 10 y§	<50	2.6 y
	50–69	0.5 y

*Based on estimates provided by Early Breast Cancer Trialists Group overview (1). Assumes the same relative treatment effect in lymph node-positive and lymph node-negative groups.

†Based on estimates provided by Cole et al. (12).

‡Assumes that the utility score associated with disease relapse is 0.5 to 0.9, compared with 1.0 for full health and 0.0 for death.

§Assumes that the same relative reduction on breast cancer mortality continues beyond 10 years with no effect on non-breast cancer deaths.

benefits beyond 10 years. If the earlier gains in survival from a reduced risk of breast cancer death were maintained after 10 years, then younger patients would gain an extra 2.9 life years and older patients an extra 0.5 year after this time. Consequently, for many women, especially younger women with lymph node-positive disease, the size of the benefit will be large enough to justify adjuvant chemotherapy. However, the situation is less clear-cut for older patients and for those at low risk of recurrence, for whom individual preference over trade-offs between survival and adverse treatment effects will be more critical.

In this setting, the individual preferences of women with early breast cancer become even more important in deciding whether such treatment is worthwhile. Preferences of women who have actually experienced the acute adverse effects of chemotherapy provide valuable information to guide decision making. Our study has shown that relatively modest survival gains would justify the adverse effects of treatment for many women. A gain in 5-year survival of at least 5% or a gain in survival by an extra year from 5 years would be sufficient to justify treatment for more than 70% of women. This means that, for most women at higher risk of recurrence, such as premenopausal women, the benefits of adjuvant CMF chemotherapy will be of sufficient size to warrant treatment. For women at lower risk, the benefit of treatment may be more questionable, and individual preferences will assume greater importance. Even for those at higher risk, individual preferences may be important. For example, 15% of women in this study would require more than 15% improvement in 5-year survival to justify therapy, an unlikely improvement in most clinical settings.

Our study identified several factors that influenced the size of the survival benefit needed. Family-related factors, such as the amount of social support available to each woman and the amount of support needed by other family members from the patient, were each associated with stronger preferences for adjuvant treatment. This suggests that for these women, the adverse effects of treatment may be of secondary importance, provided that others are available to help them or that they judged their future ability to care for others in their family to be critical. Women experiencing greater toxicity also indicated that greater survival benefit was needed. This is expected, since each patient was asked to consider 6 months of chemotherapy as similar to the treatment she had already experienced. Patients who had had a reduction in the dose of their chemotherapy also indicated that larger survival gains would be needed even after allowance had been made for treatment toxicity in an adjusted analysis. This may have reflected unrecorded toxicity. Alternatively, physicians may have been more likely to reduce dosage in a group of patients whom they assessed as less willing to accept treatment toxicity. The association with radiotherapy is less clear. Whether it reflects patient selection, an increase in unrecorded toxicity of the overall treatment, or other factors remains unknown.

This study has a number of limitations. In the patient interviews, we did not assess benefits other than survival gain associated with adjuvant chemotherapy, such as the delayed recurrence of breast cancer. The delay in recurrence will provide a small additional benefit in quality-adjusted survival and so means that the survival gains estimated in this study provide conservative estimates of the value of treatment. The study has also not taken into account psychological benefits of treatment that might possibly be gained by a sense of taking charge and feeling in control of future events. This has been identified in

other settings as one reason that patients elect to have treatment (13). A broad cross section of patients who attended a follow-up clinic were selected for the study, and almost all eligible women participated. However, these women may not be representative of all those patients considering adjuvant chemotherapy, and their views may have changed since having this treatment. It is possible that women who had chosen adjuvant chemotherapy earlier overrated its value to justify this decision. However, patients who had relapsed and who may have been less positive about their decision to have adjuvant treatment expressed similar preferences. A further limitation is that the survival rate questions did not explicitly spell out what would happen to survivors beyond 5 years. Some women may have assumed that a chance of living at least 5 years was the same as a cure, whereas others may have assumed that there would be an ongoing risk of recurrence and shortened survival after this 5-year period.

Despite its limitations, this study had some advantages over other possible designs. There was a consistency of preferences over time that was presumably related to the use of standardized interviews. The views of women who have actually experienced both the side effects of CMF chemotherapy and the concerns associated with their prognosis are also more likely to be of relevance. A number of studies using hypothetical scenarios have attempted to assess the preferences of women with early breast cancer. This method allows a range of scenarios to be considered but does not allow for the consideration of the individual woman's experience. In our study, the women's adverse experiences of adjuvant chemotherapy were used. Furthermore, the preferences of women with breast cancer may differ from others who cannot fully appreciate their unique perspective. Patient preferences for future survival may assume far greater importance once they are faced with the reality of a possible fatal outcome from cancer. Other studies support this view. For example, Galper et al. (14) found that women with invasive breast cancer considered the adverse effects of axillary lymph node dissection much less important than survival gains compared with a group of women who had had *in situ*, but not invasive, cancer. It is also possible that the women with invasive cancer have formed views that are biased in favor of the procedure so as to support their previous decision making. Another study comparing the preferences of patients with advanced cancer with those of their relatives showed that the patients considered the side effects of chemotherapy much less important than survival gains when compared with their relatives' views (5,15).

Since this study was performed, other groups have addressed similar questions. In a study of the adjuvant therapy of breast cancer, Lindley et al. (9) and Ravdin et al. (16) reported trade-offs remarkably similar to those that we observed. In other diseases, a similar willingness for patients to accept moderately or even extremely toxic therapy in return for modest survival gains has been a consistent finding (7,17–20).

The use of adjuvant chemotherapy in early breast cancer has changed substantially in the last 10 years since this study was performed. Adjuvant chemotherapy is increasingly used in women with lower risk tumours, such as those with small primary tumors and lymph node-negative tumors. It is also being used more often in older women. The nature and duration of treatment have also changed. The treatment and prevention of side effects have also improved. Anthracycline-containing chemotherapy regimens have been shown to provide additional modest benefits over CMF chemotherapy in disease-free sur-

vival (7% improvement at 5 years) and overall survival (3% improvement) (1). These additional gains come at the cost of more severe side effects than those of CMF chemotherapy. However, the side effects are shorter lived in the most commonly used anthracycline-based regimen of four cycles of treatment over the course of 3 months. Supportive therapy has also improved over the course of the last 10 years, reducing the frequency and severity of some important side effects. There are also new combinations of adjuvant chemotherapy undergoing evaluation including taxane-containing combinations and high-dose chemotherapy regimens. Further studies are needed to assess patient preferences in these settings. The evaluation of these regimens in the context of randomized trials in representative samples of patients will be of particular value. Furthermore, consideration needs to be given to undertaking such studies of women both before and after they receive such chemotherapy. This would enable one to assess the extent that preferences are altered as a result of the decisions made by women to undertake chemotherapy. These studies will need to be designed carefully to ensure that the interviews have appropriate and not unintended effects on the patient's actual decisions.

In conclusion, we believe that it is feasible to obtain individual patient preferences for adjuvant chemotherapy in early breast cancer based on an assessment of the trade-off between the adverse effects of treatment and survival gains. For younger women at high risk, the improvements in survival will usually be sufficient to justify therapy, whereas for older women and for those patients at low risk of recurrence, assessment of individual preferences may be critical to optimal decision making. Additional studies will be of considerable value in guiding the use of more recently developed adjuvant chemotherapy regimens.

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